A PROTEIN-FREE EXTENDER FOR SEMEN CRYOPRESERVATION IN WOOD BISON

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ABSTRACT

Animal proteins are a common constituent of semen extenders to protect sperm from cold shock but they raise issues concerning biosecurity and undefined composition. The purpose of this study was to determine if cholesterol-cyclodextrin (CC) can be used to replace egg yolk from extenders in cryopreservation of beef and bison semen. In initial study, semen was collected from wood bison and frozen in either conventional egg yolk (TEYG) or CC Tris-glycerol (CC-TG; 2 mg CC/mL semen) extender. Sperm motion characteristics, i.e., total motility, progressive motility, curvilinear velocity (VCL), average path velocity and straight-line velocity were determined using computer-assisted sperm analysis. There were no difference in sperm motion parameters other than VCL. Following fixed-time artificial insemination (FTAI), TEYG semen yielded 9/21 (43%) pregnancy rates while no bison conceived with 2 mg CC-TG semen. Concentration of CC used and bison species specific problems were suspected for lack of pregnancies.

In the second study, three experiments were conducted with lowered concentrations of CC in both beef and wood bison to investigate the issue of failure to conceive. In Experiment 1, semen was collected from beef bulls and frozen in TEYG, 1 mg CC-TG, or 2 mg CC-TG extender. Pregnancy rates were 20/40 (50%), 20/41 (49%), and 5/39 (13%) for TEYG, 1 mg CC-TG and 2 mg CC-TG semen following FTAI of Hereford-cross cows, respectively. In Experiment 2, 0.5 mg CC-TG extender was tested in beef cows. Pregnancy rates of 25/46 (54%), 26/46 (57%) and 13/40 (33%) for TEYG, 0.5 CC-TG, and 1 mg CC-TG semen following FTAI of Hereford-cross cows, respectively. In Experiment 3, lower concentrations of CC were tested in wood bison. Pregnancy rate of 6/11 (55%) and 4/11 (36%) were achieved for TEYG and 1 mg CC-TG semen, respectively. These were the first reports of pregnancies in bison and beef cows using CC-TG extender.

The overall results of the study demonstrated that CC can be used to effectively replace egg yolk from semen extenders for beef bull and wood bison.

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LIST OF ABBREVIATIONS

AI:	Artificial insemination
ARTs:	Assisted reproductive technologies
BSP:	Binder of sperm proteins
CASA:	Computer-assisted semen analysis
CC:	Cholesterol-cyclodextrin complex
COC:	Cumulus-oocyte complexes
°C:	Degree Celsius
EY:	Egg yolk
FITC-PNA:	Fluorescein isothiocyanate peanut agglutinin
FSH:	Follicle-stimulating hormone
hCG:	Human chorionic gonadotropin
h:	Hour
IACR:	Intact acrosome
IPM:	Intact plasma membrane
IU:	International unit
IVF:	*In vitro* fertilization
LDL:	Low density lipoproteins
LH:	Luteinizing hormone
LPC:	Lysophosphatidylcholine
µg	Microgram

µL	Microliter
mg:	Milligram
min:	Minute
mL:	Milliliter
mm:	Millimeter
Mol:	Mole
mM:	Millimole
nM:	Nanometer
n:	Number
PGF2α:	Prostaglandin
PI:	Propidium iodide
ROS:	Reactive oxygen species
TCA:	Tris-citric acid
v/v:	Volume/Volume
VAP:	Average path velocity
VCL:	Curvilinear velocity
VSL:	Straight-line velocity
ZP:	Zona pellucida

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. General introduction

Wood bison (*Bison bison athabascae*) is a distinct subspecies of the American bison and historically ranged from north western Canada extending into Alaska (Gates et al., 2001). In the late 1800s, wood bison populations experienced a sharp decline due to extensive commercial hunting and introduction of exotic bovine diseases (Flores, 1991). With European settlers, bison populations lost much of their original habitat since they were hunted as a strategy to reduce the aboriginal population. Consequently, bison population reached a low of 250 individuals in the late 1800s (Soper, 1941). Early conservation efforts led to the establishment of conservation herds around North America. Recent estimates place wild wood bison population at approximately 10,000 individuals across 12 subpopulations and is currently classified as "special concern" by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) (COSEWIC, 2013). However, conservation efforts led to the introduction of bovine diseases such as tuberculosis and brucellosis along with introgression of cattle genetics in bison herds. Diseased animals not only have decreased survival and reproductive success, they pose threats to infect wild species, domestic livestock and human population. Current disease control strategies may become inadequate with future expansion of both wild bison herds and cattle grazing land (Shury et al., 2015). Therefore, there is a pressing need to eliminate diseases from wild wood bison herds while preserving their valuable genetics.

In situ conservation of wild bison populations have been suggested to be ineffective due to cost and concerns raised by commercial livestock operations (COSEWIC, 2013). In 1990, an environmental assessment panel concluded that eradication of diseased bison herds will be the only suitable method in stemming endemic diseases (Connelly et al., 1990). Therefore, advancements in assisted reproductive technologies are required to prevent the loss of genetic diversity by harvesting gametes and propagating disease-free bison herds to repopulate. Little was known about bison reproduction until recently. Within the past decade, reproductive seasonality, ovarian dynamics and semen characteristics have been characterized (Helbig et al., 2007; Lessard et al., 2009; McCorkell et al., 2013). However, there are limited studies published in the literature

on improving cryopreservation success of bison semen. Bison share similar reproductive anatomy and physiology with domestic cattle where assisted reproductive techniques (ARTs) are used extensively and successfully. Thus, cattle ARTs can be applied as an inexpensive yet effective approach to harvest important bison genetics from disease-free animals.

To preserve male genetics, cryopreservation of semen is the most common method. Due to the limited genetic diversity within isolated wood bison populations, banking male genetic material is ideal for the movement of genetic resources from one herd to another for propagation of new populations (Comizzoli et al., 2000). Early attempts to cryopreserve ejaculated bison semen were met with lower post-thaw sperm characteristics in comparison to domestic cattle (Dorn, 1995; Lessard et al., 2009) An adjustment to the freezing curve i.e. a greater freezing rate increased bison sperm survival and demonstrated that cryopreservation can yield bison semen possessing acceptable post-thaw quality (Hussain et al., 2011).

Cryopreservation of semen is a harmful process mainly due to intracellular ice formation and cold shock during initial cooling. Intracellular ice formation during the freezing process causes significant physical and osmotic stress on sperm (Parks and Graham, 1992). Cryoprotectants (e.g. glycerol) are widely used in semen cryopreservation to bind with free water to prevent intracellular ice crystal formation. In addition, cold shock occurs during the initial cooling i.e. from room temperature to 4 °C. As the temperature decreases, sperm plasma membrane undergoes phase change (from liquid to gel state) and its integrity is disrupted resulting in increased membrane permeability and loss of major cations necessary for sperm function and survival (Quinn and White, 1966). Sperm cold shock is commonly prevented by adding egg yolk in semen extender. However, the use of animal products poses significant biosecurity concerns with the possible transmission of zoonotic diseases. Regulations on semen preparation for international transportation suggest that semen should be free of animal products (CFIA, 2002; OIE, 2016). Therefore, there is a huge pressure on domestic livestock industry and conservation groups to find alternates for animal products for semen processing. Plant based semen extender are commercially available (e.g. Andromed) and can be used to cryopreserve bovine semen (Layek et al., 2016a). However, attempts at freezing ejaculated bison semen with Andromed resulted in poor post-thaw sperm characteristics (Lessard et al., 2009).

The overall goal of this study was to solve the conundrum of semen freezing in bison and improve biosecurity by developing an animal protein-free cryopreservation method for semen. Domestic cattle were used as an animal model for wood bison. Furthermore, animal protein-free semen extenders meet regulations for international transportation and can drastically minimize associated biosecurity risks, which can be applied to the cattle industry. Successfully cryopreserved semen will allow to use assisted reproductive technologies such as embryo transfer and fixed-time artificial insemination in bison reproduction. In this study we evaluated the efficacy of exogenous cholesterol as an egg yolk replacer in bovine semen extender, and investigated the fertility potential (both *in vitro* and *in vivo*) of semen frozen in protein-free extender.

1.2. Wood bison

1.2.1. Historical perspective

To fully appreciate the extent of the bison problem, a historical view is important. Once, millions of wood bison roamed from boreal regions of north western Canada to Alaska (Gates et al., 2001; Soper, 1941). However, the actual number may have been much higher due to the smaller range estimated by Soper (IUCN, 2010). Bison populations declined at a steady rate after the arrival of European settlers but it was not until the late 1800s when the decimation of bison herds became obvious (IUCN, 2010). The indiscriminate hunting for both fur and meat was the main cause for this sharp decline (Hornady, 1889). Peace-Athabasca Delta, considered to be the core range of wood bison, was a center for fur trading by the North West Company and Hudson's Bay Company (Bradley and Wilmshurst, 2005; Ferguson, 1989). Construction of the Canadian Pacific Railway and westward expansion of settlers for arable land in the late 1800s further exacerbated the problem. By the end of the 19th century, less than 300 wood bison remained (Soper, 1941).

The Buffalo Protection Act of 1877 was the first legal policy implemented by the Canadian government to prohibit bison hunting in a response to the sharp decline of plains bison but this act remained ineffective due to lack of reinforcement (Hewitt, 1921). After the extirpation of plains bison from Canada in 1880s (Raup, 1933), members of the Mounted Police patrolled north of Peace River in 1897 for the recovery of wood bison population (Bradley and Wilmshurst, 2005; IUCN, 2010; McCormack, 1992). Wood Buffalo National Park (WBNP) was established in 1922 by the Federal Government of Canada to protect bison and indigenous populations from a large influx of trappers (Graham, 1923; McCormack, 1992).

Previous to the establishment of WBNP, Wainwright Buffalo Park (WBP) was established to protect plains bison populations in 1909. However, the park has reached at its carrying capacity by the 1920s. Due to public opposition against bison slaughter, federal government was forced to ship 6673 plains bison from WBP to WBNP between 1925 to 1928 (Carbyn et al., 1998; McCormack, 1992; Raup, 1933). As feared by biologists, interbreeding occurred between plains and wood bison resulting in hybridization. In addition, bovine tuberculosis and brucellosis were discovered in WBNP in 1937 and 1956 respectively (Connelly et al., 1990; Fuller, 1962). It was suggested that disease transmission occurred originally from domestic cattle to plains bison and thus forth into indigenous wood bison population. The controlled slaughtering from the 1950s to late 1960s to remove these diseases from wild herds was unsuccessful (McCormack, 1992). Nonetheless, bison population recovered to approximately 12,000 heads within WBNP (Soper, 1941).

In the 1960s, two successful attempts were carried out to establish disease-free wood bison herds from WBNP. Mackenzie Bison Sanctuary (MBS) was founded in 1963 by translocating 18 individuals negative for tuberculosis and brucellosis from WBNP (McFarlane Zittlau et al., 2006; Tessaro et al., 1993). From 1963 to 1968, 26 more wood bison initially tested free of diseases were moved from WBNP to Elk Island National Park (EINP) and were held in isolation for disease testing. Unfortunately, 14 animals were found to be positive for tuberculosis and brucellosis and were culled along with all adults that originated from WBNP leaving 11 neonates to be the founding population (Wilson and Strobeck, 1999; Wobeser, 2009). Both herds (MBS and EINP) remained free of tuberculosis and brucellosis. In addition, EINP herd has acted as source of animals for six additional conservation herds and private commercial herds (IUCN, 2010).

Table 1.1.

Early established wood bison herds in Canada.

Herd	Capture location	Number of founders	Herd size[c]	Disease status[d]
WBNP	-	250[a]	4500	Diseased
MBS	WBNP	18	2000	Disease-free
EINP	WBNP	11[b]	320	Disease-free

[a]Lowest estimated number of animals (Soper, 1941).

[b]Animals tested positive for tuberculosis and brucellosis are excluded.

[c]Adapted from McFarlane et al., 2006.

[d]With bovine tuberculosis or brucellosis.

Returning from the brink of extinction, there are an estimated 10,000 wood bison in conservation herds in Canada (IUCN, 2010). However, this is less than 6% of the original population and over 50% of the animals are infected with tuberculosis and brucellosis.

1.2.2. Cultural and commercial significance

Bison is an iconic animal of Canada with significant cultural and economic implications. Humans have coexisted with ancestral forms of bison approximately 13,000 years ago after crossing the Bering Land Bridge into North America before the divergence of the present two subspecies (wood bison and plains bison) 5,000 years prior (Van Zyll de Jong, 1986). Bison remains were found in 52% of the Clovis sites (early Paleo-Indian) with empirical evidence that the Clovis people were specialized large game hunters (Waguespack and Surovell, 2003). Bison hunting continued into recent time for indigenous groups and remained even in farming tribes (Bamforth, 2011). Bison remains as an important spiritual symbol in various native tribes even today. The arrival of Europeans caused a shift in human-bison interactions. Contrary to the previous seasonal bison hunting, the commercialization of bison products between European settlers and aboriginals were established on the fur trade (Innis and Ray, 1999). Later, the indiscriminate bison hunting promoted by the government was used as a tool to solve the "Indian problem" by removing their food source. Both acted to decimate the bison population. Although bison still remains indivisible to many aboriginal tribes' culture and traditions, many have

recognized the commercial value of bison. The Inter-Tribal Bison Cooperative initiated by the Lakota Sioux tribe expanded bison ranching operations for economic benefits (Goodstein, 1995).

Native tribes are not alone in the change of perspective on bison. With the movement in North America towards "natural" and healthy foods, the specialized bison meat industry has found its niche. Free range bison meat is lower in fat, calories and higher in iron and linolenic acid concentrations in comparison to beef (Marchello, 1989; Rule, 2002). Consumers are willing to pay for the increased cost of bison meat in return for nutritional benefits in comparison to other commercial meats available (Yang, 2013). In addition, further revenue can be generated through the sale of bison by-products.

As of 2007, there are around 400,000 bison in private commercial herds across Canada and the United States of America (IUCN, 2010). Annual slaughter of bison inspected by the United States Department of Agriculture nearly tripled from 17,674 heads in 2000 to 51,798 in 2015 (USDA, 2016). There were approximately 975 bison farms with 120,000 animals in Canada in 2016 (Statistics Canada, 2016). Since bison require longer time than domestic cattle to reach slaughter conditions, there is a strong push for improving production methods. Manipulation and knowledge of reproductive physiology of bison will be advantageous to industry and can drastically improve economic gain and management efficiency.

1.2.3. Current status and challenges

Wood bison conservation efforts are currently challenged by the combination of three major problems. First is the presence of endemic diseases within wild bison herds. Over 50% of wild bison herds are currently infected with bovine tuberculosis and brucellosis. Tuberculosis is caused by *Mycobacterium bovis* and is chiefly transmitted through respiratory secretion and inhalation (Stamp, 1944). *M. bovis* is mostly confined to the lungs and results in lymph node lesions in the respiratory system (Francis, 1958). Brucellosis is caused by *Brucella abortus* commonly resulting in abortion, infertility and weak newborns (Mccorquodale et al., 1985). It affects the lactation and can cause mastitis (Herrera et al., 2008). Brucellosis is primarily transmitted through mucosal contact with infected fetal and placental fluids or tissues (Carvalho Neta et al., 2010). Bulls are susceptible to infection of the reproductive tract; however, venereal transmission does not appear to play a role. Diseased animals not only experience reduced reproductive success and survival but they act as disease carriers to infect healthy animals

including other species and domestic livestock. It has been suggested that bison may be the only wildlife reservoir of these two diseases (Shury et al., 2015). Detection of tuberculosis in Hook Lake in 2006 after 10 years of intense testing and isolation led to the eradication of the entire herd (Himsworth et al., 2010) demonstrated the resiliency and difficulty associated with disease management and eradication.

Limited genetic diversity and introgression of plains bison genetics into wood bison populations is another problem. With the overcrowding of plains bison population in Wainwright National Park, the Canadian government, due to public pressure, transported 6,000 plains bison to the WBNP in 1925 (IUCN, 2010). This decision led to the hybridization of plains bison with the already limited numbers of wood bison. As seen in Table 1.1., disease-free wood bison in conservation herds were established with only 29 founder animals. Therefore, the majority of genetic diversity is found within WBNP. However, there is little movement of genetics between herds due to concerns over disease transmission. Inbreeding depression is also a major concern in the long term survival of wood bison populations but difficult to determine due to the lack of pedigrees (Hedrick, 2009). Inbreeding depression was documented in one bison herd with 5 founders where after 100 years, the herd showed a 3 year increase in average age and 67% natality rate (births per cow) compared to other herds over a period of 6 years (Hedrick, 2009). Wood bison face the same problem as Canadian Holsteins where increased inbreeding coefficient resulted in decreased milk production and survival rate (Thompson et al., 2000). Furthermore, genetic drifts act to exacerbate inbreeding depression with the majority of conservation herds isolated from each other and in small populations.

It could be said that a pure wood bison does not exist and its classification as a subspecies is debatable. Although there are microsatellite differences between wood and plains bison herds, plains bison genetics exist in all wood bison herds (Geist, 1991). Further, genetic studies showed that wood and plains bison exhibit less difference than between various cattle subspecies and does not support the distinction as subspecies (Cronin et al., 2013; Cronin and Cockett, 2009; Halbert et al., 2005; Stormont et al., 1961). However, there are phenotypic differences among bison subspecies in spite of inhabiting the similar habitat and remaining in persistent discrete groups. Recently, COSEWIC recognized wood bison as a distinct designateable unit, unique from plains

bison for the purposes of conservation (COSEWIC, 2013). Exchange of genetic material between herds, WBNP mainly, will be essential to the continuing growth of wood bison population.

Eradication of endemic diseases while preserving genetic diversity should be the overall objective for bison conservation. Unfortunately, socio-political problems act as a final major obstacle. Although the desire for bison conservation is common to various agencies; however, the coordination and cooperation between agencies and stakeholders are often difficult. Provincial and federal governments share conflicting views on the management and control of bison herds. It is likely that the establishment of a separate committee is necessary to facilitate the conservation efforts. This committee can direct funds more efficiently and establish long term goals of bison conservation. Involvement of the private industry is ideal but objectives in conservation often differ if not contradict with the commercial sector. Policies involving conservation of wild bison populations should avoid artificial selection on traits that improve commercial production such as docility and meat yield.

Lastly, gaining public trust and support will be vital. Possible conservation strategies include vaccination and selective culling but these measures will be difficult to implement on the entire herd and can be expensive (Shury et al., 2015). An alternative strategy was proposed by The Federal Environmental Assessment and Review Office's (FEARO) proposal to eradicate all diseased individuals in WBNP and preserving genetics through gamete salvage techniques. However, this was met with public outcry, and was never implemented (Connelly et al., 1990). With depopulation of diseased animals followed by repopulation with disease free herds being the most effective option, major advancements have been made to understand and advance the assisted reproductive technologies in wood bison in the past decade. Not only does extraction of genetic material from diseased herds circumvent the issues concerning transporting a diseased animal. Gametes can be washed to remove infectious agents. In addition, cryopreservation allows for storage and transport of important genetic material and help to propagate genetically diverse herds. Similar to the wide usage of semen cryopreservation in the domestic livestock industries, cryopreservation of bison sperm can assist in conservation efforts and allow for the application of other ARTs.

1.3. Semen cryopreservation

Cryopreservation is process where biological material (cells, tissues or organs) are cooled to below 0 °C and preserved in liquid nitrogen (-196 °C) for long-term storage. It is generally assumed that biochemical processes are suspended at ultra-low temperature due to the non-availability of free water for cell metabolism. Cells face osmotic, chemical and physical stresses resulting in various degrees of cellular damage throughout cryopreservation (Medeiros et al., 2002). Successful cryopreservation of semen has allowed advancements in assisted reproductive techniques such as artificial insemination, embryo transfer and *in vitro* fertilization. These procedures are widely utilized in the livestock industry today.

1.3.1. Principles of semen cryopreservation

Semen cryopreservation may be divided into three distinct processing steps. The first step is extension or dilution in which sperm are diluted in a suitable medium called extender. The addition of penetrating cryoprotectants in extender results in rapid osmotic (volume) changes in mammalian sperm. The second step involves the initial cooling of sperm to 4 °C in which their plasma membranes undergo phase transition changes from liquid to gel state. The third step is a combination of freezing and thawing in which cells face osmotic and physical challenges associated with ice crystal formation and osmotic shock. For successful cryopreservation, the development of suitable extender and freezing rate very are important.

Cryoprotective effects of glycerol were accidentally discovered (Polge et al., 1949). Since then, glycerol has been widely used in mammalian semen cryopreservation. Glycerol, being hygroscopic, binds to intracellular free waters within sperm to minimize ice crystal formation, which would otherwise damage cell structures. However, glycerol imposes cytotoxic and osmotic stresses on cells due to it being a penetrating cryoprotectant. Deleterious effects of glycerol to sperm cytoskeleton and mitochondria have also been reported (Macías García et al., 2012; Pursel et al., 1978). The underlying mechanisms are yet unknown; however, the toxic effect may be minimal under most cryopreservation and ARTs protocols. The concentration of glycerol is required to be optimized for each species to offer the greatest cumulative protective effect with minimum osmotic and cytotoxic shocks. Human sperm are more resistant against osmotic stress in comparison to bull sperm (Guthrie et al., 2002), therefore, greater concentration of glycerol can be used for human extender (15% v/v) compared to bovine extender (7% v/v) (Brotherton, 1990).

Glycerol addition can occur in one-step protocol before the initial dilution, or delayed until after cooling in a two-step protocol. The beneficial effects of two-step glycerol addition have been observed in rams and boar sperm (Almlid and Johnson, 1988; Gil et al., 2003). In cryopreservation of bovine semen, the one-step protocol is commonly practiced due to its ease.

Initial cooling of sperm from room temperature to 4 °C results in phase transition changes in the sperm plasma membrane. Phase transition changes can result in decrease in membrane asymmetry which lead to irreversible damage (Anzar, 2017). These damages during initial cooling are collectively called as "cold shock". The consequences of cold shock will be explored below.

Between -5 to -15 °C, extracellular ice crystals begin to form, extruding the solutes and thus increasing extracellular osmolality. Water is subsequently drawn out from the intracellular space leading to high intracellular osmolality before cells freeze. This process called the "solution effect" and is critical to reduce intracellular ice formation and depends on freeze rate. If the freeze rate is too rapid, it will not allow complete removal of intracellular water and ice crystal formation can damage cell organelles. If the freeze rate is too slow, then sperm keep on dehydrating, eventually leading to membrane damage (Woelders, 1997).

Similar events in reverse are experienced by sperm during thawing. Fast warming is suggested to minimize reformation of intracellular ice crystals. In addition, sperm experience rapid increase in volume during the rehydration process. Thawing at 75 °C for 7 seconds significantly reduced acrosome reacted populations in equine sperm (Bradford and Buhr, 2002). Fast warming resulted in greater sperm velocities after 2 hours compared to thawing at 37 °C in dairy semen (Muino et al., 2008). However, there was no effect of warming on *in vivo* fertility. Damage sustained by sperm from the process of cryopreservation is cumulative; previous membrane conformation changes persist after warming (Anzar, 2017). Minimizing damage to the sperm membrane can improve the success of cryopreservation.

1.3.2. Sperm plasma membrane

Plasma membrane of sperm is unique in comparison to that in most somatic cells. The cell membrane acts as a physical barrier to maintain cell homeostasis. Fluctuations in sperm intracellular ionic balance, or loss and disorientation of membrane proteins can result in irreversible physiological changes detrimental to sperm fertilization potential. Sperm plasma

membrane can typically be subdivided into three functional components (domains): head, midpiece and tail. The head of sperm holds the nucleus. In addition, the apical portion of sperm head carries Golgi-derived acrosome, responsible for the penetration of the oocyte's zona pellucida (ZP) after sperm-oocyte binding. Sperm midpiece houses mitochondria for energy production. Tail is a long flagellum necessary for sperm movement.

1.3.3. Lipid bilayer

As proposed by Singer and Nicholson in 1972, the plasma membrane is composed of a two dimensional mosaic of phospholipids, proteins and cholesterol (Singer and Nicolson, 1972). The plasma membrane acts as a physical barrier between extracellular and intracellular milieus. The fluid mosaic model postulates the existence of two phases: liquid crystalline and gel phases. The liquid crystalline phase has increased fluidity and allows increased lateral movement of lipids in comparison to the gel phase. The state of the lipid bilayer is dependent on the lipid composition and the temperature (Cullis et al., 1986). Within the range of normal physiological temperature (~37 °C), sperm membranes are mostly arranged in the lamellar composition (Fig. 1.1.). However, during initial cooling from room temperature to 4 °C, a decrease in temperature results in phase transition of non-bilayer preferring phospholipids from hexagonal II to the lamellar formation (Murata and Los, 1997). The hexagonal II conformation increases permeability to ions in comparison to the lamellar conformation (Cullis et al., 1986). Phase transition from liquid to gel state of the lipid bilayer can result in leaky membranes allowing increased permeability of ions across the plasma membrane and change the protein orientation. Spectroscopic studies have demonstrated that cold shock damage during initial cooling is due to lipid phase transition of plasma membrane (Drobnis et al., 1993). Phase transition in bovine sperm occurs between 20 °C to 10 °C (Parks and Graham, 1992; Sieme et al., 2015).

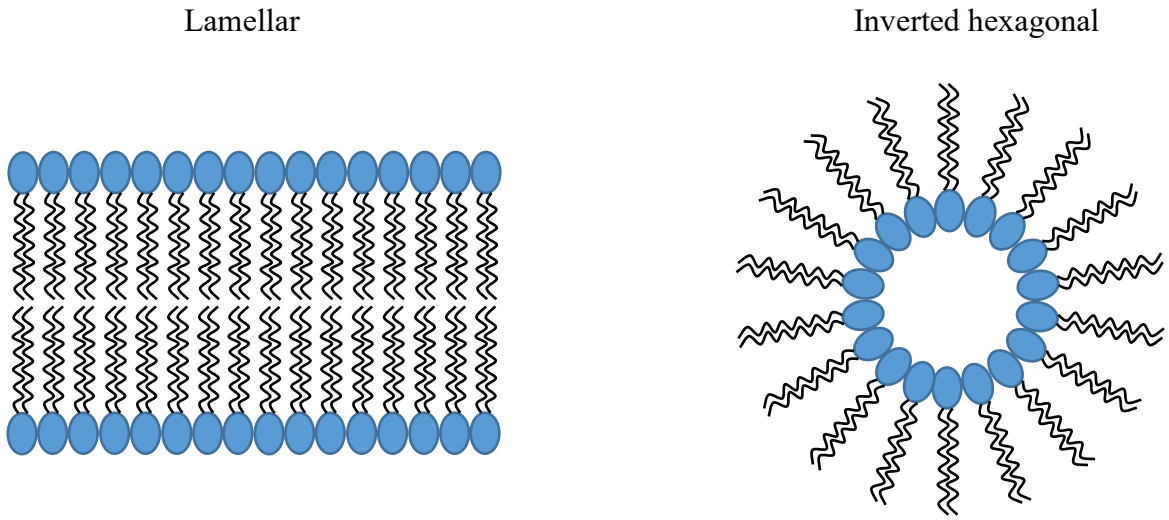


Fig. 1.1. Representative organization of lipids in lamellar and inverted hexagonal phases.

1.3.4. Membrane proteins

During spermatogenesis, the majority of cytoplasm along with cellular organelles such as endoplasmic reticulum and golgi apparatus is extruded from sperm in the form of residual bodies. Therefore, sperm lack the mechanisms for protein and lipid synthesis, and transport. In addition, sperm are required to undergo a series of sequential events within the female reproductive tract, namely capacitation and acrosome reaction, prerequisites for fertilization. These events are highly regulated and are localized in specific regions/domains of sperm membrane. During capacitation, the membrane fluidity of sperm head at the acrosomal region increases (Brucker and Lipford, 1995). Disruption in the sperm membrane integrity causes irreversible damage to the sperm fertility potential.

Sperm capacitation is an essential mechanism to occur before acrosome reaction and is controlled by both external and internal factors. Capacitation can be generally defined as the process where sperm plasma membrane undergo modifications and attain the fertilization capacity. However, once capacitated, sperm have decreased survival time (longevity) compared to un-capacitated counterparts. Sperm capacitation involves the removal of decapacitation factors (proteins) from sperm plasma membrane. Binder of sperm proteins (BSPs) found in the seminal plasma initially bind to the sperm membrane upon ejaculation, preventing premature capacitation. BSPs removal by heparin-like compounds in the oviductal fluid release sperm from oviduct in a

wave-like fashion (Bailey, 2010). In addition, cholesterol are displaced along with BSPs from the plasma membrane and act as the trigger for capacitation (Bailey, 2010). Addition of cholesterol *in vitro* to human sperm prevents acrosome reaction to occur (Renee J Zarintash and Cross, 1996). Reduction in membrane cholesterol to phospholipid ratio over time will increase membrane permeability leading to calcium influx and subsequent capacitation and acrosome reaction (Cross, 1998). Intracellular calcium ion (Ca^{2+}) in bull sperm increased from 25 nM to 160 nM following capacitation conditions (Breitbart et al 1985). Influx of Ca^{2+} induces downstream protein tyrosine phosphorylation possibly allowing zona pellucida (ZP) recognition (Bailey, 2010). In addition, the influx of intracellular Ca^{2+} enables acrosome reaction which triggers fusion of the outer acrosomal membrane and inner leaflet of plasma membrane to release hydrolytic enzymes (e.g. hyaluronidase and acrosin) to digest the cumulus cells and ZP (Flesch and Gadella, 2000). The exact mechanism of extracellular Ca^{2+} entering into the cell during sperm capacitation is still unclear. Inhibition of Ca^{2+} ATPase which normally removes intracellular Ca^{2+} could potentially lead to buildup intracellular Ca^{2+} (Flesch and Gadella, 2000). Structural changes to sperm membrane result in irreversible membrane and ionic imbalance that as a result reduces sperm survival and fertility potential.

1.4. Semen extenders

Semen extender serves two major functions i.e. it increases the volume and extends the survival of semen. Extending the volume of semen is critical to increase the production of semen doses from a single ejaculate of high genetic value sire or where access to the intact bull is limited. Although many extenders are commercially available, their major function is to protect sperm from damages caused by cryopreservation.

Maintaining optimal pH and osmolality of semen extenders are two fundamental requirements of sperm survival. Buffering part of semen extender should withstand the acidic waste produced by highly metabolic sperm. Tris-citric acid buffer (TCA), a combination of Tris base and citric acid, is the buffer of choice for semen extension in bulls in majority of breeding stations (Purdy, 2006). In addition, fructose is commonly provided as a metabolic energy source. Glycerol, as discussed previously, is critical to protect sperm against deep freezing (Bailey et al., 2000). Other permeating cryoprotectants such as ethylene glycol and DMSO have also been used

for bovine semen freezing (Swelum et al., 2011; Tasdemir et al., 2013). However, glycerol remains the most commonly used cryoprotectant for semen cryopreservation.

Ever since the discovery of egg yolk as a protective agent against cold shock during initial cooling (Phillips and Lardy, 1940), it has been used extensively in semen extenders. Low density lipoproteins (LDL) have been identified as protective agents within egg yolk (Pace and Graham, 1974). It has been demonstrated that the phospholipids in egg yolk are integrated into the sperm plasma membrane to provide membrane stability (Quinn et al., 1980). Another hypothesis suggest that LDLs bind with and sequester BSP proteins in seminal plasma to prevent premature efflux of cholesterol and phospholipids (Bailey, 2010), and thus prolong sperm survival. Additionally, egg yolk contain known anti-oxidants, tryptophan and tyrosine, which can reduce ROS build up. Cholesterol, another major active component of egg yolk, has also showed a decrease in sperm susceptibility to cold shock (A Darin-Bennett and White, 1977).

1.4.1. Issues concerning egg yolk extenders

The majority of commercially available bovine semen extenders require or contain egg yolk (20% v/v). Milk protein based extenders containing casein micelles are also available. However, animal proteins (egg yolk or milk) based semen extenders face two major concerns: i) composition of animal protein based extenders can vary from batch to batch, and ii) biosecurity risk associated with animal products (de Ruigh et al., 2006). Although chicken egg yolk is normally used; levels of cholesterol, phospholipids and polyunsaturated fatty acids can differ depending on the source (Grobas et al., 2001). Hen fed with additional sunflower oil showed a significant increase in polyunsaturated fatty acids in egg yolk (Foulkes et al., 1980). Although there was no difference in post-thaw sperm motility in bulls, the varied composition is unreliable. More importantly, the use of animal products poses biosecurity risks significantly. The current use of antibiotics in semen extenders could further exacerbate the issue of antibiotic resistance. Reduced effectiveness of the conventional antibiotic mixture (gentamicin, tylosin, spectinomycin, and lincomysin) have been observed for bull semen (Gloria et al., 2014). Egg yolk can also act as a carrier for zoonotic diseases such as avian flu, salmonella, *Escherichia coli*, and mycoplasma (Layek et al., 2016b). In addition, no difference was detected in bacterial load between pure egg yolks, commercial egg yolk-based diluents in powder or liquid form, being the most contaminated

(Bousseau et al., 1998). The necessity for disease-free extenders is more urgent when it concerns the conservation of endangered (both wild and domestic) species.

1.4.2. Plant based semen extenders

Alternatives to the conventional egg yolk extender have been available to overcome the growing biosecurity concerns. Plant-based soybean lecithin extenders have been used with varied success to freeze semen. In dairy bulls, improved post-thaw characteristics and similar fertility rate with lecithin extender in comparison to egg yolk extender was reported (Gil et al., 2000). However, there are contrary reports where lecithin-based extender resulted in lower sperm longevity and field fertility (Muiño et al., 2007; van Wagtendonk-de Leeuw et al., 2000). Low fertility rates were observed with lecithin in stallion but it appeared to be a suitable alternative for buck semen (Andréa et al., 2013; Chelucci et al., 2015; Papa et al., 2011). Further research is necessary to investigate the effectiveness of soybean lecithin extenders. However, lecithin faces similar issues like egg yolk with its inconsistent composition from batch to batch.

1.4.3. Cholesterol

Cholesterol plays a critical and concentration-dependent role in membrane stability and permeability. Cholesterol is composed of structurally rigid steroid rings (Fig. 1.2) and rigidity is important in membrane stabilization. Cholesterol content in plasma membranes is significantly higher (> 30% mol) compared to membranes surrounding intracellular organelles (< 12% mol) (van Uitert et al., 2010). This difference in cholesterol content is reflective of the necessity of a low permeability physical separation from the extracellular environment. High concentration of membrane cholesterol is known to increase the order of lipids, membrane stability and decreased membrane fluidity (Bloom et al., 1991). Since membrane permeability is also dependent on temperature, cholesterol also has a two prong effect to counteract damages due to temperature fluctuations. At higher temperatures where the sperm membrane is in the liquid phase, cholesterol act to bind tightly to lipids and increase membrane rigidity (Oldenhof et al., 2015). In contrast, at lower temperatures where the plasma membrane is in the gel phase, cholesterol prevents the phase transition from the lamellar phase to the inverted hexagonal conformation and thus again increases membrane rigidity (Wolkers et al., 2002).

Cholesterol to phospholipid (C/P) ratio of sperm can be an indicator of the protective effects of cholesterol. The C/P molar ratio of domestic livestock species were 0.26, 0.36 and 0.45 in boar, stallion and bull sperm, respectively (Parks and Graham, 1992). Bull sperm have higher percentage of post-thaw survival compared to boar and stallion semen. Although some differences could be attributed to artificial selection of "good freezers" within the bovine industry, boar semen is sensitive to initial cooling due to low cholesterol content. Removal of cholesterol from the plasma membrane is required for initiating capacitation. Capacitation was arrested by cholesterol loss despite high intracellular Ca^{2+} levels from incubation with calcium ionophore (Cross, 1998). The C/P ratio of seminal plasma from sub-fertile stallion was 2.5 times greater in comparison to fertile stallions (Brinsko et al., 2007). Decreased capacity for capacitation and acrosome reaction will reduce sperm fertility potential.

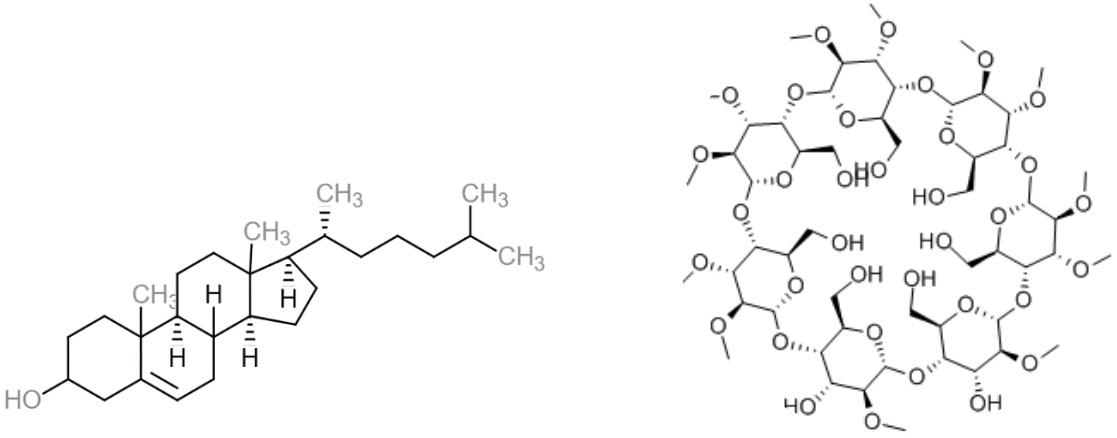


Fig. 1.2. Molecular structure of cholesterol (left) and methyl-β cyclodextrin (right). Cyclodextrins contain a hydrophobic (non-polar) center, cholesterol acceptor. The peripheral regions (polar) bind with plasma membrane and deliver cholesterol to cell membranes in, under aqueous conditions.
http://www.chemicalbook.com/ChemicalProductProperty_US_CB5696215.aspx]

1.4.4. Cholesterol-cyclodextrin complexes

Cyclodextrins are cyclic oligosaccharide with hydrophobic center and hydrophilic exterior. Cyclodextrins are commonly used in pharmaceutical industry as a drug delivery system. They are

capable of incorporating non-polar molecules such as cholesterol and act as a carrier to transport these molecules within aqueous solutions. Methyl-β-Cyclodextrin has particular affinity for cholesterol (cholesterol-cyclodextrin complexes; CC) and have been shown to deliver cholesterol onto plasma membranes (Klein et al., 1995).

As discussed previously, cholesterol plays a critical role in modulating membrane stability and protects the sperm against phase transitional changes. The pre-exposure of CC before the dilution of semen with egg yolk extender improved post-thaw sperm motility with success in bulls, bison and boars (Hussain et al., 2013; Lee et al., 2015; Purdy and Graham, 2004a). In addition, increasing CC concentration increases the concentration of cholesterol incorporated into the sperm membrane (Lee et al., 2015). However, to our knowledge, CC alone has not been reported yet to protect sperm from cold shock.

Table 1.2.

Major findings involving cholesterol-cyclodextrin complexes and egg yolk for cryopreservation of sperm from different mammalian species.

Source	Year	Species	Major findings
Klein et al.	1995	Guinea Pig	- CC can supplement myometrial plasma membranes with cholesterol.
Combes et al.	2000	Stallion	- Improved post-thaw sperm motilities with CC treatment in EY extender.
Purdy and Graham	2004a	Bull	- Cholesterol incorporated into all parts of the sperm membrane after incubation. - Improved post-thaw sperm viability with CC treatment in EY extender.
Purdy and Graham	2004b	Bull	- Sperm treated with CC can undergo capacitation and acrosome reaction.
Moore et al.	2005	Stallion	- Greater sperm membrane cholesterol concentration in CC treated group post-thaw
Tomás et al.	2011	Boar	- Improved osmotic tolerance with CC treated sperm
Hussain et al.	2013	Bison	- Improved post-thaw sperm motilities with CC treatment in EY extender - Optimal CC concentration between 1 and 3 mg/100x^{6} sperm

1.5. Semen assays

It is generally accepted that approximately 50% of sperm die after cryopreservation (Watson, 2000). Examination of sperm quality is important to assess viability and fertility potential. Parameters such as sperm motility patterns, plasma membrane and acrosome integrity, and binding to oocyte zona pellucida help in determining the fertility potential of semen.

1.5.1. Sperm motion characteristics

Sperm motion characteristics are some of the most important indicators of sperm quality and are commonly measured in breeding soundness examination of bulls. Manual visual assessment can be conducted under the microscope to determine the percentage of motile sperm. However, this method can be tedious and is subject to human bias.

Computer-assisted semen analysis (CASA) was developed to offer an objective analysis of sperm motion by tracking the movement of the sperm head (Dott and Foster, 1979). Aside from total and progressive motilities, CASA is able to provide information on sperm velocities including straight line velocity (VSL, speed of sperm head from start to finish in a straight line); average path velocity (VAP, speed of sperm head following its average path of movement); and curvilinear velocity (VCL, speed of sperm head following its actual track). Sperm velocities could be a more effective indicator of sperm quality compared to motilities (Defoin et al., 2008).

1.5.2. Flow cytometric evaluation of plasma membrane integrity and acrosome reaction

Fluorescent dyes used in combination with flow cytometer can be used to assess sperm structures (intact plasma membrane and intact acrosomes). Flow cytometer analyses each sperm individually and allows the rapid quantification of sperm structures. Intact plasma membrane, critical for sperm cell viability, can be determined using permeating dyes such as propidium iodide (PI). PI binds to DNA and will not permeate an intact plasma membrane. If integrity of the plasma membrane is compromised, PI enters the sperm and fluorescing red when excited with a 488 nm laser (Anzar et al., 2002).

Acrosome integrity is often assessed with the aid of lectin-conjugated fluorescent dyes. After acrosome reaction or if the acrosome is damaged, lectin gain access and bind to the inner or outer membrane of acrosome. Fluorescein isothiocyanate (FITC) is commonly conjugated to peanut agglutinin (PNA; lectin) and will emit green light when excited with a 488 nm laser (Silva and Gadella, 2006). The combination of these two dyes will permit the concurrent analysis of sperm plasma membrane and acrosome integrity.

1.5.3. *In vitro* fertilization

Perhaps the most indicative parameter for sperm fertility is through field fertility trials. However, due to restrictions in resources and access to animals, *in vitro* fertilization (IVF) can be

used as a good indicator of sperm penetrating ability to oocytes. IVF enables the usage of desirable parents without the transportation of live animals or to salvage important genetics. Resulting embryos can be readily cryopreserved or implanted into a synchronized recipient. Since heparin is conventionally added to the fertilization media, capacitation is normally induced within 4 h of incubation and fertilization typically occurs within 8 h (Bailey, 2010; Parrish, 2014). However, this does not mimic *in vivo* environment where sperm is required to travel up the oviduct and sperm longevity becomes an issue.

1.6. Assisted reproductive technologies in bison

Wood bison, unlike domestic cattle, are seasonal breeders; the ovulatory season extends from middle of July to March (Dorn, 1995; Vervaecke and Schwarzenberger, 2006). Follicular development in wood bison cows was characterized recently by serial ovarian ultrasonography and authors reported that wood bison exhibit follicle development in a wave like fashion similar to that described for domestic cattle (McCorkell et al., 2013). Ovulatory capacity in wood bison was attained when the dominant follicle of a wave reached ≥10 mm, and could be electively induced for the purposes of synchronization using hCG (Palomino et al., 2015). There has been no previous report on pregnancy rate in wood bison following fixed-time artificial insemination using frozen semen.

Bison and domestic cattle belong to the Family Bovidae, and have the same number of chromosomes (2n = 60). Thus, they have similar physiologic and anatomic characteristics. Although difficult, concerted efforts to produce bison/cattle crosses have produced hybrids. Hybridization attempts were later abandoned due to low birth rate of fertile offspring. However, this led to the eventual introgression of cattle genetics into bison. These similarities provide impetus to attempt to apply assisted reproductive technologies developed in cattle, such as semen cryopreservation, estrus synchronization and artificial insemination, to bison. Epididymal sperm from plains bison have been frozen with up to 60% post-thaw progressively motility (Aurini et al., 2009). However, access to epididymal sperm is limited to tissues salvaged after death and results in the collection of low numbers of sperm. Electroejaculated bison sperm have been frozen with reasonable post-thaw success in both wood and plains bison (Lessard et al., 2009). Greater sperm survival was achieved by using a fast freeze rate (-40 ºC/min) and by exposing bison sperm to CC before dilution in conventional semen extenders containing egg yolk (Hussain et al., 2013, 2011).

Bison semen did not freeze well in plant-based semen extender (Andromed); hence, conventional EY extender remains the only option (Lessard et al., 2009).

In this thesis, we hypothesized that the supplementation of bovine sperm with exogenous cholesterol will replace egg yolk from semen extender. The overall objective of this study was to cryopreserve bovine semen without including egg yolk in semen extender.

1.7. General hypotheses and objectives

Hypothesis 1: Cholesterol-cyclodextrin complexes can replace egg yolk in semen extenders for cryopreservation of bison semen.

Objective 1: To compare the post-thaw quality of bison sperm frozen with egg yolk vs cholesterol-cyclodextrin complexes semen extender.

Hypothesis 2: Bison semen cryopreserved after treatment with cholesterol-cyclodextrin complexes in the absence of egg yolk can yield acceptable fertility *in vitro* and *in vivo*.

Objective 2: To determine the fertility potential of bison sperm frozen without egg yolk *in vitro* and *in vivo*.

Hypothesis 3: High concentration of cholesterol-cyclodextrin ≥ 2 mg/mL sperm has a negative impact on *in vivo* fertility as a result of premature induction of the acrosome reaction in frozen-thawed bison sperm.

Objective 3: To investigate if sperm frozen in 2 mg cholesterol-cyclodextrin Tris-glycerol extender have poor post-thaw characteristics and fertility potential.

Hypothesis 4: Low concentrations of cholesterol-cyclodextrin (0.5 mg/mL, 1 mg/mL) are beneficial for sperm during cryopreservation.

Objective 4: To determine the post-thaw characteristics and *in vivo* fertility of beef and bison sperm frozen without egg yolk using lower concentrations of cholesterol-cyclodextrin.

CHAPTER 2: FERTILITY POTENTIAL OF BISON SEMEN FOLLOWING CRYOPRESERVATION WITHOUT EGG YOLK

2.1. Abstract

The purpose of this study was to develop an egg yolk-free semen extender for the cryopreservation of wood bison semen. Semen was collected from four wood bison bulls by electroejaculation and the pooled bison ejaculates were divided into two aliquots. Semen in aliquot 1 was diluted with conventional egg yolk (TEYG) extender. Semen in aliquot 2 was first treated with exogenous cholesterol (2 mg CC/mL semen) and then diluted with glycerolated extender without egg yolk at either 22 °C or 4 °C (CC-TG extender). Both kinds of semen (TEYG and CC-TG) underwent programmed freezing and were stored in liquid nitrogen. In Experiment 1, sperm motion and structural characteristics of frozen thawed semen were determined at 0 and 2 h post-thaw using a computer-assisted sperm analyzer and a flow cytometer. Post-thaw sperm total motility, progressive motility, average path velocity, curvilinear velocity and straight-line velocity, plasma membrane integrity and acrosome integrity at 0 h and change in these characteristics over 2 h, were similar (NS) between TEYG and CC-TG extenders. The temperature at which glycerol was added had no effect on the post-thaw sperm motion and structural characteristics. In Experiment 2, the *in vitro* fertility potential of bison semen frozen in TEYG and CC-TG extenders was determined using a heterologous *in vitro* fertilization assay with bovine oocytes. Cleavage and blastocyst rates, assessed on Days 2 and 8 (Day 0 = fertilization) respectively, did not differ (NS) between TEYG and CC-TG extender. In Experiment 3, the *in vivo* fertility potential of TEYG and CC-TG extended bison semen was tested in synchronized female bison. Following artificial insemination, TEYG semen yielded 43% fertility rate whereas CC-TG semen did not produce any pregnancy. In Experiment 4, effect of extender on plasma membrane and acrosome integrity was determined to investigate the absence of pregnancies using CC-TG extender. Sperm in TEYG and CC-TG extender were incubated with and without lysophosphatidylcholine for 6 h to investigate if spontaneous acrosome reaction due to cryocapacitation occurs. Acrosome reacted sperm did not differ between TEYG and CC-TG extender regardless of LPC treatment. In conclusion, *in vitro* results clearly demonstrated that bison semen can be successfully frozen without egg yolk and further studies are required to achieve pregnancy in bison.

2.2. Introduction

Semen cryopreservation is a proven assisted reproductive technology to conserve male genetics for a long time and to exploit the genetic potential of superior sires through artificial insemination. It is also used for the distribution of male genetics between geographically isolated herds. A major limitation in artificial insemination with cryopreserved bison semen is its marginal post-thaw quality (Dorn, 1995). Advancements within the last decade in bison semen cryopreservation led to acceptable post-thaw motility in epididymal and electroejaculated sperm (Aurini et al., 2009; Hussain et al., 2013; Lessard et al., 2009). During initial cooling from room temperature to 4 °C, sperm plasma membrane undergoes phase separation due to aggregation of hexagonal II forming phospholipids which leads to disorientation of proteins (Sieme et al., 2015). Animal products such as egg yolk and milk are commonly used in semen extenders to protect sperm against phase separation by replenishing lost membrane lipoproteins (Parks and Graham, 1992). However, animal proteins (originated from egg yolk and milk) raise biosecurity concerns associated with the possible transmission of zoonotic diseases. Usage of pathogen-free semen diluents are emphasized by various health regulation agencies including the Canadian Food Inspection Agency (CFIA, 2002) and World Organisation for Animal Health (OIE, 2016). Therefore, alternatives to animal proteins in traditional semen extenders are a priority to minimize the biosecurity risks for international trade.

Cholesterol plays an important role in modulating membrane stability. Reduced freezing ability of bison sperm may be related to lower membrane cholesterol content in comparison to domestic cattle (Dorn, 1995). Exogenous cholesterol can be incorporated into the hydrophobic center of β-cyclodextrins forming cholesterol-cyclodextrin complexes (CC), and cholesterol can be delivered into the sperm plasma membrane (Klein et al., 1995). The sperm exposure to CC prior to dilution in traditional extenders containing egg yolk increased sperm survival in cattle and bison semen (Hussain et al., 2013; Purdy and Graham, 2004b). Since addition of cholesterol can improve membrane stability and protect sperm against phase transition changes during initial cooling, CC may be able to replace animal products (e.g., egg yolk) in semen extenders.

The specific objectives of the present study were to determine the post-thaw quality and fertilization potential (*in vitro* and *in vivo*) of bison semen cryopreserved using cholesterol-cyclodextrin complexes in place of egg yolk. The study design permitted investigation of the

protective effects of exogenous cholesterol during the initial cooling phase transition by adding glycerol at room temperature (22 °C) or refrigerated temperature (4 °C); and to determine fertility in bison using different ovarian synchronization protocols and fixed-time artificial insemination.

2.3. Materials & Methods

All chemicals used in this study were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise mentioned.

2.3.1. Animals

Wood bison bulls (n = 4) used in this study were kept under uniform conditions of management and nutrition, at the Native Hoofstock Centre near Saskatoon, Saskatchewan, Canada (52° 10'N/106° 38'W). Animals were habituated to a special hydraulic chute system prior to experimentation to minimize stress (McCorkell et al., 2013). Animal procedures related to semen collection, estrus synchronization and fixed-time artificial insemination were approved by the Animal Care Committee, University of Saskatchewan, Saskatoon, Canada (Animal Use Protocol # 20100150).

2.3.2. Experiment 1: Effects of CC-TG extender and the temperature at which glycerol was added on the post-thaw characteristics of bison sperm

Semen was collected from four wood bison bulls by electroejaculation (Pulsator IV Auto Adjust; Lane Manufacturing Inc. Denver, CO, USA), twice weekly, during the breeding season (October and November). A total of 12 ejaculates were pooled for processing across 5 collection days. Semen frozen in Experiment 1 was used in all subsequent experiments.

Preparation of TEYG and CC-TG extenders

Conventional TEYG extender

Tris-citric acid (TCA) buffer containing Tris base (3.03%, w/v), citric acid monohydrate (1.74%, w/v) and fructose (1.2%, w/v) in milli-Q distilled water (pH 7.1) was initially prepared. The conventional TEYG extender was prepared by adding glycerol (7%, v/v), EY (20%, v/v), gentamycin sulfate (500 µg/mL), tylosin (100 µg/mL), and lincomycin / spectinomycin (300/600 µg/mL) in TCA buffer. The final extender was centrifuged at 12,000 x g for 15 min at 4 °C. The

supernatant was recovered in 50 mL Falcon tubes and stored at -20 °C. TEYG extenders were warmed to 37 °C before use.

CC-TG extender

Cholesterol-cyclodextrin was prepared as previously described (Purdy and Graham, 2004a). In brief, cholesterol (200 mg, Cat. C8667) was dissolved in 1 mL chloroform (Solution A). Methyl β-cyclodextrins (1 gm, Cat. C4555) was dissolved in 2 mL methanol (Solution B). Solution A (0.45 mL) was added to Solution B (2 mL) and the mixture (CC) was mixed until it became homogenous. The CC mixture was then poured into a glass petri dish and dried under a gentle stream of nitrogen gas. The resulting crystals were allowed to dry overnight in a desiccator and stored at 22 °C in a glass bottle. The working solution was prepared by dissolving 50 mg of CC in 1 mL TCA buffer on the day of semen collection, at 22 °C. Egg yolk-free extender, Tris-glycerol (TG, 2X concentration) was prepared in TCA buffer with glycerol (14%, v/v), gentamycin sulfate (1 mg/mL), tylosin (200 µg/mL), lincomycin / spectinomycin (600/1200 µg/mL). The TG extender was stored at -20 °C and thawed before use.

Experimental design

Raw semen was kept at 32 °C and transported to the laboratory within 2 h of collection. Initial sperm motility and concentration were evaluated using a computer-assisted sperm analyzer (CASA, SpermVision 3.5, Minitube Canada, Ingersoll, Ontario), described in detail below. On each day of collection, ejaculates with a sperm concentration of $\geq 200 \times 10^6$/mL and sperm total motility of ≥60% were pooled to minimize the effect of bull-to-bull differences. Due to difficulties with animal handling or poor ejaculate quality, a total of 12 ejaculates were pooled across 5 collection days.

Each pooled semen sample was divided into 2 aliquots (Fig. 2.1). Semen in aliquot 1 (TEYG extender, n = 5, n is the number of pooled ejaculates) was diluted in a one-step dilution to 50×10^6 sperm/mL with TEYG extender at 37 °C and kept at room temperature (22 °C) until the other aliquot was ready. For preparation of aliquot 2 (CC-TG extender), components were added sequentially. Semen in aliquot 2 was diluted to 100×10^6 sperm/mL with TCA buffer at 37 °C, cooled to 22 °C in 20 min and treated with 2 mg CC/mL semen for 15 min. To investigate the effect of temperature at which glycerol was added on post-thaw characteristics of bison sperm,

CC-treated semen was further divided into 2 sub-aliquots. In sub-aliquot A (pre-cool; glycerol addition at 22 °C; n = 4), the semen was diluted with TG extender at 22 °C. In sub-aliquot B (post-cool; glycerol addition at 4 °C; n = 4; one sample was lost), the semen remained as such. Both sub-aliquots A and B were then cooled to 4 °C. After cooling, semen in sub-aliquot B was diluted with TG extender. Final concentration of semen was $50x10^6$ sperm/mL. Final composition of CC-TG extender was CC (1 mg/mL), glycerol (7%, v/v), gentamycin sulfate (500 µg/mL), tylosin (100 µg/mL), and lincomycin / spectinomycin (300/600 µg/mL) in TCA buffer. All semen samples were chilled to 4 °C in a walk-in cooler for 90 to 120 min and packaged into 0.5 mL French straws. Semen was frozen using a programmable cell freezer (ICE-CUBE 14-S, Sy-Lab Version 1.30, Gerate GmbH, Neupurkdersdof, Austria) to -80 °C with a previously established freezing curve; i.e., -3 °C/min from 4 °C to -10 °C and -40 °C/min from -10 °C to -80 °C (Hussain et al., 2011). Semen straws were held at -80 °C for 5 min before plunging them into liquid nitrogen. Semen straws were thawed at 37 °C for at least 1 min and evaluated with CASA within 5 minutes. Sperm total motility (TM), progressive motility (PM), average path velocity (VAP), curvilinear velocity (VCL) and straight-line velocity (VSL) were analyzed initially upon thawing, using following CASA settings for bison semen (Lessard et al., 2009). Semen samples (2.5 µL) were loaded onto a warm (37 °C) chamber slide and 7 fields were analyzed with approximately 200 sperm per field. Thawed semen was held at 37 °C for additional 2 h and analyzed to assess sperm longevity (stress test).

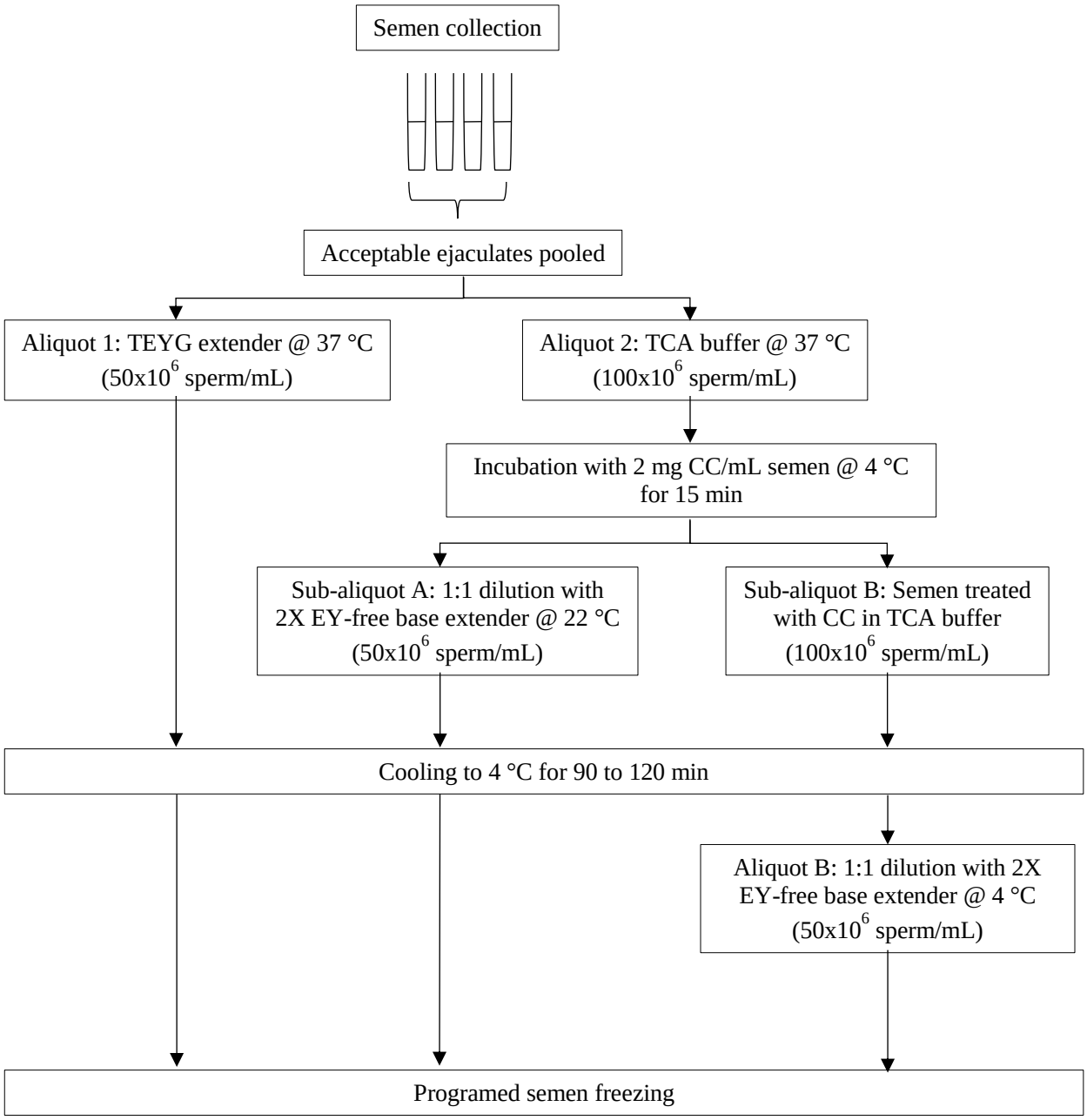


Fig. 2.1. Semen processing procedures for cryopreservation of bison semen with egg yolk or cholesterol-cyclodextrin complexes extender and the addition of glycerol pre- or post-cooling (Experiment 1). Semen was collected from wood bison bulls (n = 4) weekly for 5 collections.

Flow cytometer analysis

Bison sperm plasma membrane and integrity of acrosome were assessed simultaneously following the procedure developed in our laboratory (Anzar et al., 2011). Frozen-thawed bison semen (TEYG or CC-TG extender with glycerol addition at 22 °C) was layered on 4 mL of 45% Percoll in 15 mL conical centrifugation tubes and centrifuged at 750x g for 15 min to remove extender portion. The supernatant was discarded and sperm were re-suspended in 10 mL sp-TALPH (100 mM NaCl, 3.1 mM KCl, 0.3 mM NaH_2PO_4, 0.4 mM $MgCl_2$, 40 mM HEPES, 0.4 mM EDTA, 21.6 mM lactate, 1 mg/mL PVA, 2 mM $CaCl_2$, 1 mM pyruvate, 10 mM $NaHCO_3$) and centrifuged at 500x g for 5 min to remove Percoll particles. Resulting supernatant was discarded and sperm concentrations were determined with a haemocytometer. Sperm in pellets were then diluted to $1x10^6$ sperm/mL with sp-TALP (100 mM NaCl, 3.1 mM KCl, 0.3 mM NaH_2PO_4, 0.4 mM $MgCl_2$, 10 mM HEPES, 0.4 mM EDTA, 21.6 mM lactate, 2 mM $CaCl_2$, 1 mM pyruvate, 25 mM $NaHCO_3$, 6 mg/mL BSA). The semen samples were then incubated at 39 °C and 5% CO_2 for 0 and 2 h. Following fluorescent dyes were added per ml sperm suspension: 1 µL fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA; Sigma chemicals, St. Louis, MO; stock 1 mg/mL PBS) for acrosome integrity and 6.25 µL propidium iodide (PI; Invitrogen; stock 2.4 mM in water) for plasma membrane integrity. Semen-dye mixture was incubated at room temperature (22 °C) in the dark for 20 min. Sperm were fixed by adding 10 µL of 10% formaldehyde in each semen sample.

At least 10,000 sperm of each semen sample were analyzed with flow cytometer (Partec Cyflow Space, version 2.4 by Partec GmbH, Münster, Germany) equipped with a 400 mW argon laser. FITC-PNA and PI were excited with 488 nm blue laser and their emission spectra were detected with photo-multiplier detectors at FL-1 and FL-3, respectively. The data were acquired by FloMax software (version 2.4) provided by Partec GmbH. All samples were passed through the flow cytometer with a speed of 1 µL/s.

Forward- and side-light scatters were used to identify the sperm. Simultaneous fluorescence data of both PI and FITC-PNA were recorded on log scales. Sperm which did not stain with PI had an intact plasma membrane whereas the remaining PI-stained sperm were considered to have a compromised plasma membrane (CPM). Similarly, sperm which remain unstained with FITC-PNA had an intact acrosome whereas FITC-PNA stained sperm had a

compromised acrosome. Two dimensional PI/FITC-PNA data yielded four sperm subpopulations in different quadrants (Q1-Q4; Fig. 2.1). Compensations were conducted to minimize the overlap of emission spectra of different fluorophores using FloMax software. The sperm population with an intact plasma membrane and intact acrosome (Q1; PI^-/FITC-PNA^-) was used in Experiment 1.

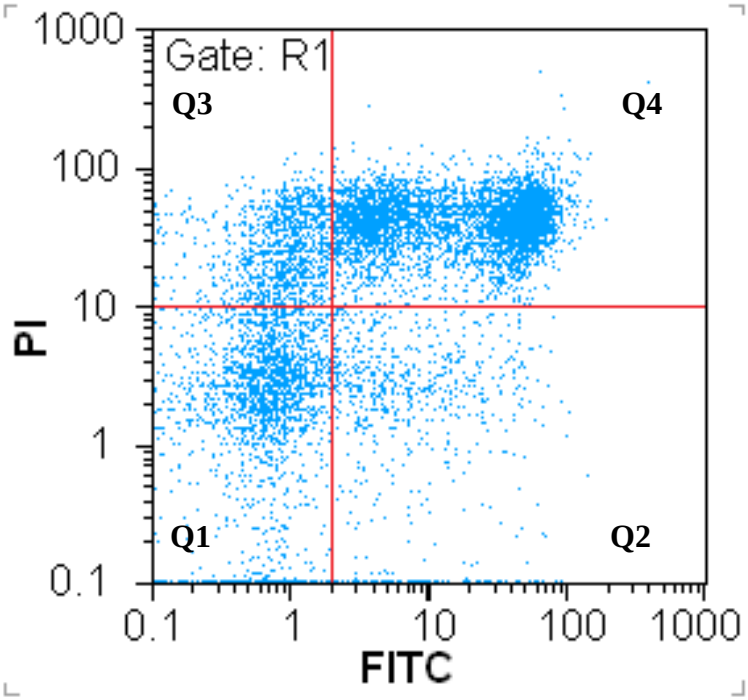


Fig. 2.2. Example of flow cytometer analysis of post-thaw integrity of the plasma membrane and acrosome of bison sperm. Q1 (PI^-/FITC-PNA^-): sperm with an intact plasma membrane and acrosome; Q2 (PI^-/FITC-PNA^+): sperm with an intact plasma membrane and reacted acrosome; Q3 (PI^+/FITC-PNA^-): sperm with a compromised plasma membrane and intact acrosome; Q4 (PI^+/FITC-PNA^+): sperm with a compromised plasma membrane and reacted acrosome.

2.3.3. Experiment 2: Heterologous *in vitro* fertilization assay

Due to limited access to bison oocytes, a heterologous *in vitro* fertilization (IVF) assay was conducted using cattle ovaries procured from a local slaughterhouse. *In vitro* maturation, fertilization and embryo culture were performed as previously described (Prentice et al., 2011). Cumulus-oocyte complexes (COC) were aspirated from follicles and identified and sorted under a stereomicroscope (10x). COC with uniform cytoplasm and $\geq$3 layers of cumulus cells were

selected for further processing. The COC were washed three times in maturation medium (5% calf serum, 0.5 µg/mL FSH, 5 µg/mL LH and 0.05 µg/mL gentamycin in TCM199). Approximately 20 COC were pipetted into a 100 µL droplet of maturation medium and incubated under mineral oil at 38.5 °C, 5% CO_2 in air, for 22 h. Semen from dairy bulls collected and cryopreserved at a Canadian breeding station served as an internal control whereas dead dairy semen was used as a control for parthenogenesis. After thawing, semen samples were centrifuged through Percoll gradients (45% and 90%) in a 15 mL conical centrifuge tube and diluted to $3x10^6$ sperm/mL with Brackett-Oliphant (BO) fertilization medium (Parrish, 2014). Dairy semen for parthenogenesis then underwent rapid freeze-thaw cycles in liquid nitrogen to ensure that all sperm cells were killed. Mature COC were washed (3x) with BO containing 10% (w/v) bovine serum albumin (BSA), placed in 100 µL droplets of BO with sperm (300,000/droplet) from TEYG (n = 278, n is the number of oocytes), CC-TG (n = 299), dairy (n = 235) or dead dairy (n = 83) semen, and incubated at 38.5 °C, 5% CO_2 in air, for 18 h. Day of IVF was considered as day 0. After incubation, the presumptive zygotes were denuded by repeated pipetting and placed into 100 µL droplets of culture media (CR1aa + 5% v/v calf serum) under mineral oil for incubation at 38.5 °C, 5% CO_2, 90% N_2 and 5% O_2. Cleavage and blastocyst rates were evaluated on day 2 and 8 (post-IVF) respectively and calculated based on the number of oocytes submitted to IVF. This experiment was conducted in 5 replicates.

2.3.4. Experiment 3: Fixed-time artificial insemination of wood bison

The experiment was designed to test the effects of semen preparation (TEYG vs CC-TG) and synchronization protocol (follicle ablation vs steroid treatment) on pregnancy rate after fixed-time artificial insemination in bison during the ovulatory season (November). Female wood bison (n = 48) at random stages of the estrous cycle were assigned randomly to two synchronization groups involving transvaginal ultrasound-guided follicular ablation or intramuscular administration of estradiol and progesterone. In the follicular ablation group (n = 24), follicles ≥5 mm were aspirated with a disposable 18 gauge x 2.5 cm vacutainer needle (BD Medical, Mississauga, ON, Canada) attached to 10 mL syringe with silicon tubing (60 cm long x 1.14 mm internal diameter; Cole-Palmer, Montreal, Quebec, Canada) through the vaginal wall guided by an intravaginal convex-array ultrasound probe (Aloka SSD 900, Tokyo, Japan; Palomino et al., 2014). In the steroid-treated group (n = 24), bison were treated intramuscularly with a combination of

estradiol-17ß (2.5mg) and progesterone (50 mg) in canola oil. A progesterone-releasing intravaginal device (PRID, Vetaquinol Inc., Lavaltrie, Quebec, Canada) was inserted on the day of ablation or steroid treatment (Day 0). Progesterone devices were removed and bison were given a luteolytic dose of PGF2α (Estrumate, 500µg *im*) on Day 5 in the follicle ablation group or Day 8 day in the steroid-treated group (Fig. 2.2). Human chorionic gonadotropin (hCG, 2500 IU) was given intramuscularly 48 h after PGF2α treatment to induce ovulation. Half of the bison in each synchronization group were assigned randomly to be inseminated with bison semen frozen with TEYG or CC-TG (22 °C glycerol addition) extender. The bison were inseminated twice 12 h apart; i.e., at 24 and 36 h post-hCG treatment.

The ovaries were examined by transrectal ultrasonography using a 7.5 MHz linear-array transducer at the time of hCG treatment and at the time of the second insemination to record the size of the largest follicle and ovulation (sudden disappearance of the dominant follicle). If ovulation was not detected at the second insemination, the ovaries were examined again 12 h later. Pregnancy diagnosis was made 42 to 44 days after the last insemination by detection of a fetus with a heartbeat by transrectal ultrasonography.

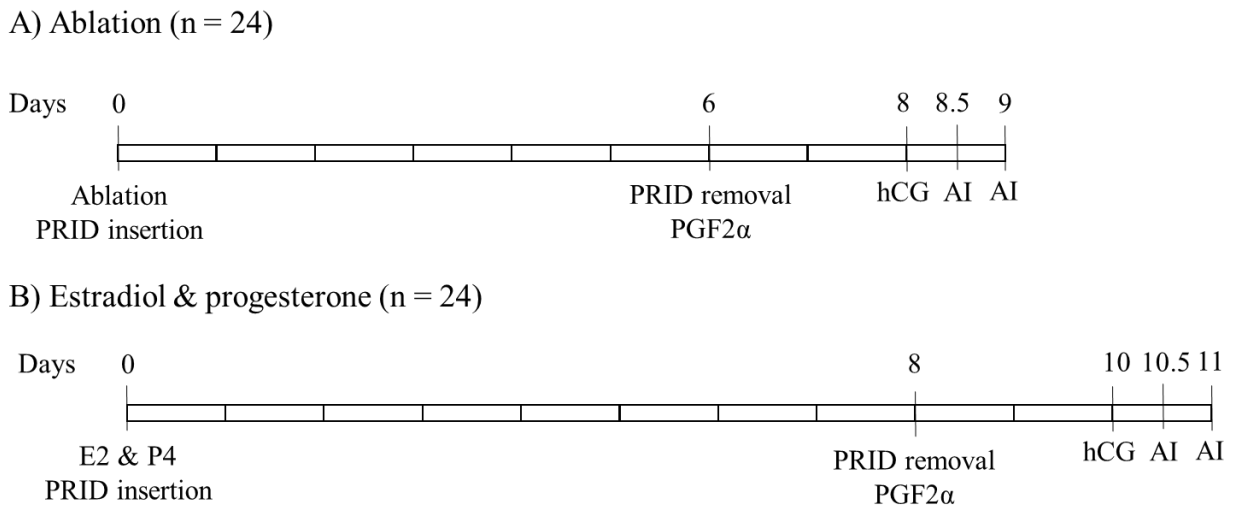


Fig. 2.3. Fixed time artificial insemination protocols in wood bison (Experiment 3).

2.3.5. Experiment 4: Spontaneous acrosome reaction in TEYG and CC-TG extended bison semen

To investigate the cause of failure in pregnancy in synchronized bison inseminated with egg yolk-free semen, this experiment was designed to assess the spontaneous acrosome reaction as a result of cryocapacitation (Knobbe et al., 2008) in both kinds of semen. Sperm plasma membrane and acrosome reaction were analysed through flow cytometry. Frozen-thawed bison semen (TEGY or CC-TG extender with 22 °C glycerolization) were processed similar to Experiment 1 except that semen samples were incubated at 39 °C and 5% CO_2 for 6 h. At 0, 4 and 6 h, two 500 µL semen samples were removed from each kind of semen. Lysophosphatidylcholine (100 µg/mL) or Sp-TALP (equal volume) was added in these samples and incubated for additional 30 min. After incubation, 5 µL of PI and 1 µL FITC-PNA were added in each sample. The mixture was incubated at 22 °C in the dark for 20 min before the addition of 10 µL of 10% paraformaldehyde to fix sperm. Flow cytometer analysis was conducted as in Experiment 1. All four sperm populations: intact plasma membrane and intact acrosome (IPM-IACR; PI^-/FITC-PNA^-), intact plasma membrane and reacted acrosomes (IPM-RACR; PI^-/FITC-PNA^+), compromised plasma membrane and intact acrosomes (CPM-IACR; PI^+/FITC-PNA^-) and compromised plasma membrane and reacted acrosome (CPM-RACR; PI^+/FITC-PNA^+) were detected (Fig. 2.1.). All sperm populations were used in this experiment, for data analysis.

2.3.6. Statistical analysis

Values are expressed as mean ± SEM unless otherwise stated. In Experiment 1, post-thaw sperm characteristics were compared among groups using repeated measures analysis of variance. In Experiments 2 and 3, binomial data (cleavage and blastocyst rates, pregnancy rate) were compared among groups by binomial generalized linear mixed model analysis of variance. In Experiment 3, dominant follicle diameter at the time of hCG treatment and interval to ovulation from hCG treatment were compared among groups using analysis of variance. In Experiment 4, data on different sperm populations were analyzed using repeated measure analysis of variance. If $P < 0.05$, the means were separated with Tukey's test. Data analyses were performed using the R Project for Statistical Computing Package (R version 3.3.1, R Foundation for Statistical Computing, Vienna, Austria).

2.4. Results

2.4.1. Experiment 1: Effects of CC-TG extender and the temperature at which glycerol was added on the post-thaw characteristics of bison sperm

Data for effect of extender and temperature of glycerol addition within CC-TG extender are shown in Table 2.1 and 2.2. Temperature of glycerol addition did not demonstrate significant effect on any sperm motion and structural characteristics within CC group. Therefore, sperm motion characteristics data for CC-TG extenders were combined to be compared against TEYG extender. Post-thaw sperm total motility, progressive motility, average path velocity, curvilinear velocity and straight-line velocity, intact plasma membrane and acrosomes (IPM-IACR) did not differ between TEGY and CC-TG bison semen. There were no interactions between semen extender and time. However, there was a significant decline ($P < 0.05$) in sperm motion characteristics from 0 to 2 h. The CC-TG extended semen showed minimum background particulates under the CASA microscope in comparison to the TEYG extended semen where yolk granules were clearly visualized.

Table 2.1.

Comparison of sperm motion characteristics (mean ± SEM) in bison semen diluted in 2 mg CC-TG extender with glycerol addition at 22 °C and 4 °C, and decline (*Δ*) in sperm motion characteristics from 0 to 2 h incubation (Experiment 1).

Sperm characteristics		Treatment*	
		Pre-cooling (Glycerol addition at 22 °C; n = 4)	Post-cooling (Glycerol addition at 4 °C; n = 5)
Total motility (%)	0 h	46±5.2[x]	43±3.4[x]
	2 h	17±3.7[y]	15±2.7[y]
	Δ	*29±4.0*	*28±2.2*
Progressive motility (%)	0 h	37±4.9[x]	35±3.5[x]
	2 h	9±2.8[y]	7±1.5[y]
	Δ	28±*3.7*	28±*2.4*
Velocity - average path (VAP; µm/s)	0 h	54±3.6[x]	53±4.1[x]
	2 h	33±2.2[y]	30±2.0[y]
	Δ	22±*1.9*	23±*2.9*
Velocity - curvilinear (VCL; µm/s)	0 h	109±8.2[x]	108±9.6[x]
	2 h	63±6.3[y]	58±6.4[y]
	Δ	46±*4.1*	50±*6.1*
Velocity - straight line (VSL; µm/s)	0 h	40±3.5[x]	36±3.3[x]
	2 h	26±2.1[y]	23±1.3[y]
	Δ	14±*2.0*	13±*2.3*

[xy]Within columns, 0 h and 2 h values are different ($P < 0.05$).

*No significant differences between temperatures of glycerol addition for any characteristic.

Table 2.2.

Comparison of sperm motion characteristics (mean ± SEM) in bison semen diluted in TEYG and 2 mg CC-TG extender, and decline (*Δ*) in sperm motion characteristics from 0 to 2 h incubation (Experiment 1).

Sperm characteristics		Treatment		P-value
		TEYG (n = 5)	2 mg CC-TG (n = 9)	
Total motility (%)	0 h	47±5.9^{x}	44±2.8^{x}	NS
	2 h	21±3.4^{y}	16±2.1^{y}	
	Δ	*26±4.0*	*29±0.8*	*NS*
Progressive motility (%)	0 h	36±5.8^{x}	36±2.8^{x}	NS
	2 h	8±3.0^{y}	8±1.5^{y}	
	Δ	*28±4.4*	*28±2.1*	*NS*
Velocity - average path (VAP; µm/s)	0 h	50±2.6^{x}	54±2.6^{x}	NS
	2 h	31±1.2^{y}	31±1.5^{y}	
	Δ	*19±2.3*	*23±1.8*	*NS*
Velocity - curvilinear (VCL; µm/s)	0 h	90±6.6^{ax}	109±6.1^{bx}	0.03
	2 h	48±4.1^{ay}	60±4.3^{by}	
	Δ	*41±4.6*	*48±3.8*	*NS*
Velocity - straight line (VSL; µm/s)	0 h	39±1.6^{x}	38±2.3^{x}	NS
	2 h	26±0.9^{y}	24±1.2^{y}	
	Δ	*13±1.7*	*13±1.5*	*NS*
Sperm with intact plasma membrane and intact acrosome (IPM/IACR)	0 h	31±5.0^{x}	34±4.5^{x}	NS
	2 h	21±3.9^{y}	27±2.7^{y}	
	Δ	*9±2.1*	*8±3.4*	*NS*

^{xy}Within columns, 0 h and 2 h values are different ($P < 0.05$).

^{ab}Within rows, values with different superscripts are different ($P < 0.05$). P-value belongs to repeated measure analysis of variance showing the overall effect of treatments on sperm characteristics.

2.4.2. Experiment 2: Heterologous *in vitro* fertilization assay

Following *in vitro* fertilization of cattle oocytes, bison semen frozen in TEYG, 2 mg CC-TG extenders, and commercially produced dairy semen (internal control) yielded 139/278 (50%), 120/299 (40%) and 122/235 (52%) cleavage rates and 49/278 (18%), 50/299 (17%) and 50/235 (22%) blastocyst rates, respectively. Cleavage and blastocyst rates did not differ between TEYG and CC-TG extenders (NS).

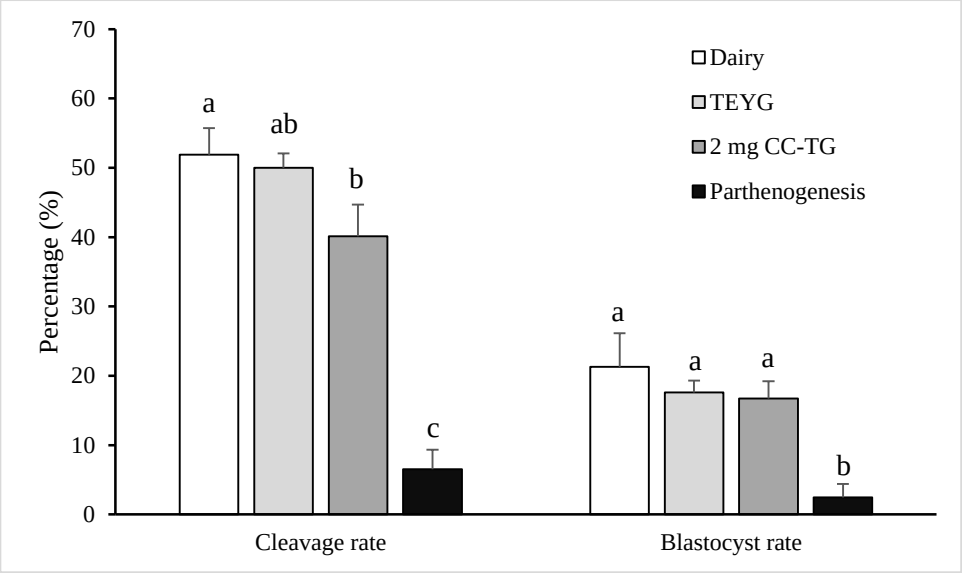


Fig. 2.4. Cleavage and blastocyst rates following heterologous *in vitro* fertilization of cattle oocytes with bison semen frozen with TEYG (n = 278 oocytes) or CC-TG (2 mg CC/mL semen; n = 299 oocytes) extenders. Cleavage and blastocyst rates (%) were expressed out of the total number of oocytes used for *in vitro* fertilization. Letters a-c denote significant difference among semen types within end points (Experiment 2).

2.4.3. Experiment 3: Fixed-time artificial insemination of wood bison

Frozen semen quality used for insemination was confirmed using CASA. Nine bison were excluded from the experiment because they appeared to have already entered the anovulatory season; neither ovulation nor a corpus luteum detected during the course of the experiment. The diameter of the dominant follicle at the time of hCG treatment was greater in the steroid-treated group than in the ablation group ($P < 0.05$; Table 2.3). The time interval from hCG treatment to

ovulation was not different between synchronization groups (P = 0.22). The proportion of bison that ovulated within 48 h after hCG treatment was 12/19 (63%) and 19/20 (95%) in the ablation and steroid-treated groups, respectively (P = 0.22). Distribution of ovulation timing is shown in Table 2.3. The majority of the animals ovulated within 36 h from hCG treatment for both the ablation and estrogen & progesterone protocol. In addition, no effect of interactions between synchronization protocols and semen extender was detected. Therefore, the data on pregnancy rates were pooled over synchronization protocols to analyse the effect of semen extender on pregnancy rates. Overall, semen frozen in TEYG extender yielded 43% pregnancy rate while no bison cow conceived with 2mg CC-TG extender.

Table 2.3.

Ovarian response in wood bison synchronized by follicular ablation (n = 19) or by intramuscular administration of estradiol and progesterone (n = 20; Experiment 3).

End points	Ablation	E2 & P4	Combined
Dominant follicle diameter (mm)	10.7±0.7[a]	14.8±1.6[b]	12.8±1.2
Time to ovulation (h)	35.3±1.8	32.2±1.6	33.5±1.2

[ab]Within rows, values with different superscripts are different (P < 0.05)

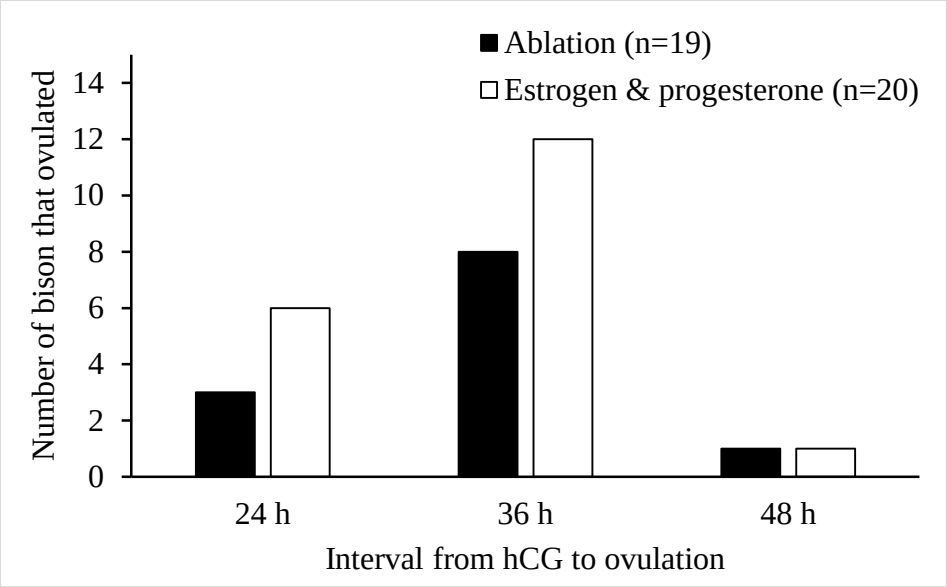


Fig. 2.5. Effect of synchronization protocol on ovulation timing from hCG treatment in wood bison (Experiment 3).

Table 2.4.

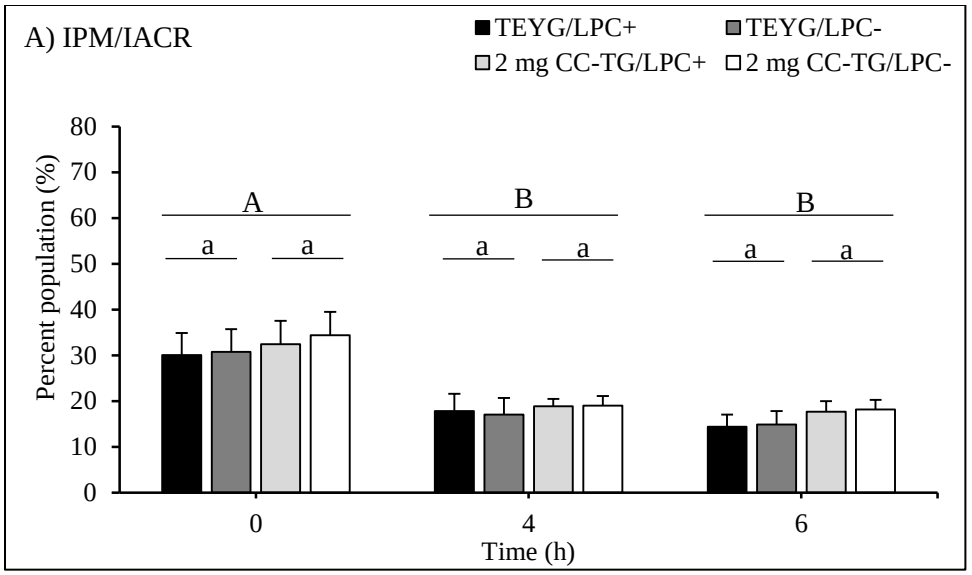
Effect of synchronization protocol and semen extender on the pregnancy rates following fixed time artificial insemination in wood bison (Experiment 3).

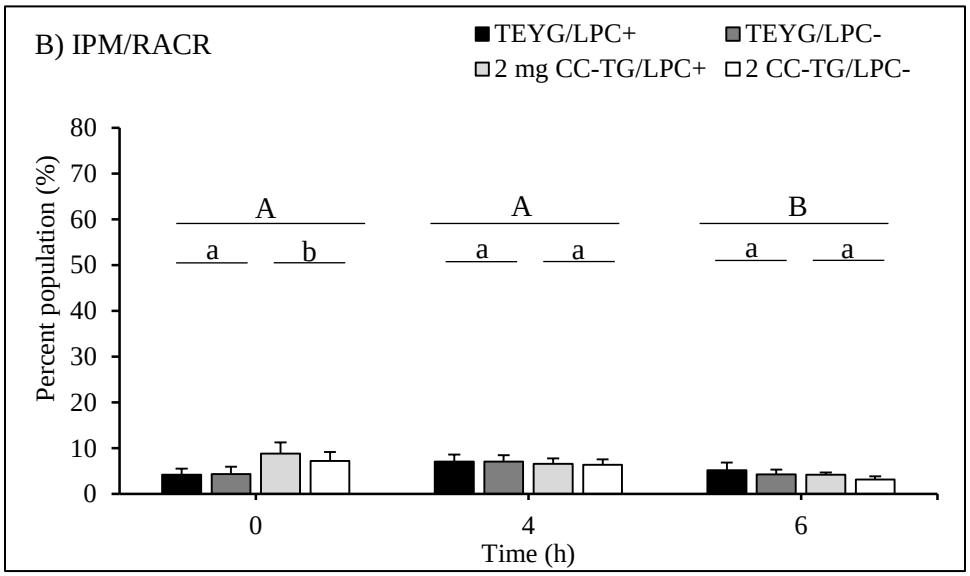
Semen extender	Synchronization protocol		
	Ablation	Estrogen & Progesterone	Total
Egg yolk	3/10 (30%)^{x}	6/11 (55%)^{x}	9/21 (43%)^{x}
Cholesterol-cyclodextrin	0/9 (0%)^{y}	0/9 (0%)^{y}	0/18 (0%)^{y}
Total	3/19 (16%)	6/20 (30%)	9/39 (23%)

Means in a column with different superscripts (x,y) denote significant difference in pregnancy rate among semen types ($P < 0.05$).

2.4.4. Experiment 4: Spontaneous acrosome reaction in TEYG and CC-TG extended bison semen

Flow cytometer data of four sperm populations are presented in Fig. 2.6. There was no significant difference in all structural characteristics due to extender at 0 and 2 h. The change in these sperm populations also did not differ due to extender. During 6 h incubation, sperm population IPM-IACR declined by 16% and CPM-RACR increased by 13% averaged over extender. TEYG extended bison semen had greater starting values accompanied by a smaller decline in comparison to conventional extender. Acrosome integrity of samples treated with LPC did not differ from samples without LPC.


A) IPM/IACR
TEYG/LPC+
TEYG/LPC-
2 mg CC-TG/LPC+
2 mg CC-TG/LPC-
Percent population (%)
80
70
60
50
40
30
20
10
0
A
B
B
a
a
a
a
a
a
0
4
6
Time (h)


B) IPM/RACR
TEYG/LPC+
TEYG/LPC-
2 mg CC-TG/LPC+
2 CC-TG/LPC-
Percent population (%)
80
70
60
50
40
30
20
10
0
A
A
B
a
b
a
a
a
a
0
4
6
Time (h)

(Continued)

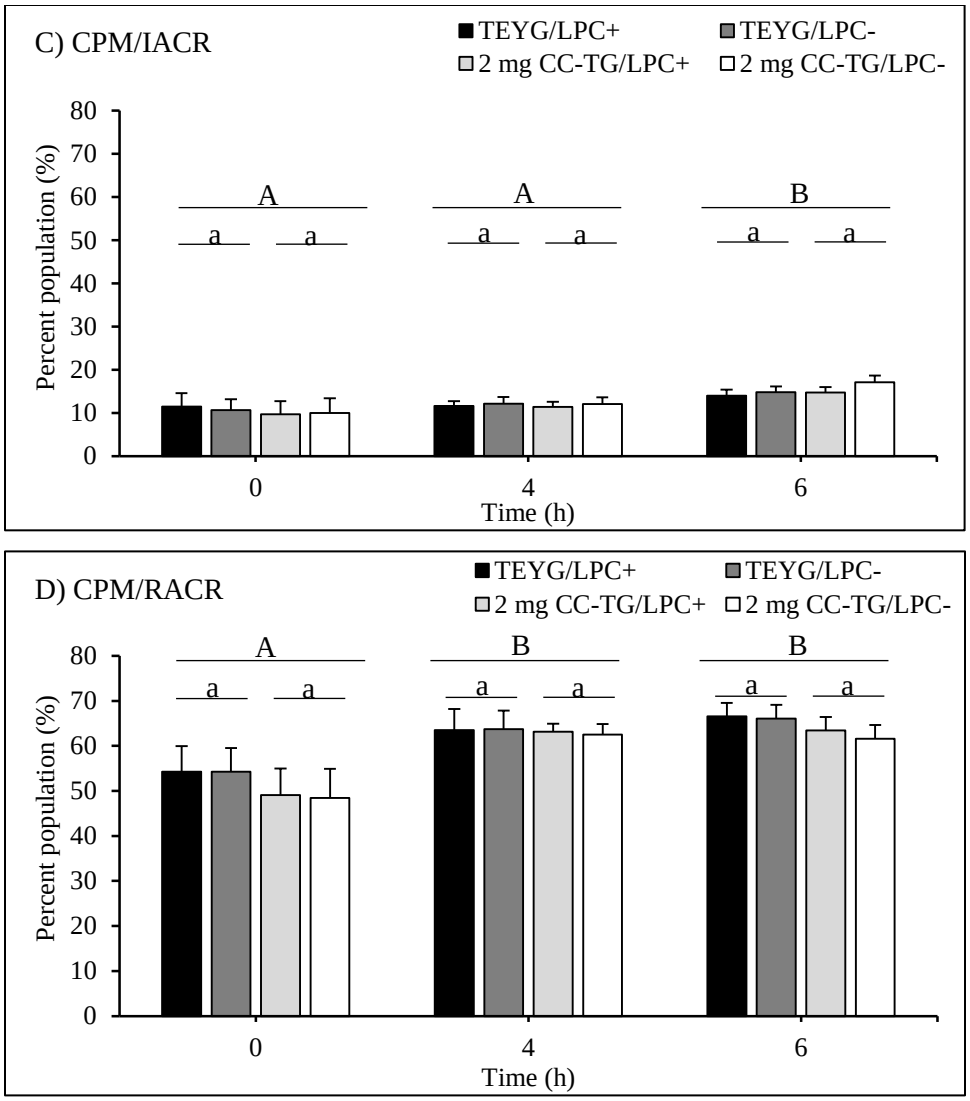


Fig. 2.6. Sperm spontaneous acrosome reaction dynamics in EY and CC extended bison semen, after incubation with or without LPC at 4 and 6 h (n = 6 frozen semen samples [replicates] processed on different days; Experiment 4). Letters A-B denote significant difference among time and letters a-b denote significant difference among extenders.

Abbreviations: EY^+, semen with egg yolk; EY^-,semen without egg yolk; LPC^+, addition of lysophosphatidylcholine; LPC^-, no addition of lysophosphatidylcholine; IPM/IACR, sperm with intact plasma membrane and intact acrosome (Q1, PI^-/FITC-PNA^-); IPM/RACR, sperm intact plasma membrane and reacted acrosome (Q2, PI^-/FITC-PNA^+); CPM/IACR, sperm compromised plasma membrane and intact acrosome (Q3, PI^+/FITC-PNA^-); CPM/RACR, sperm with compromised plasma membrane and reacted acrosome (Q4, PI^+/FITC-PNA^-). Within a specific time, no difference between treatment groups.

2.5. Discussion

Cryopreservation of bison semen in CC-TG extender was successful when treatment with 2 mg CC/mL semen was carried out before cooling. Post-thaw sperm characteristics and *in vitro* fertilization assay outcomes of CC-TG extended bison semen were comparable to the TEYG semen. This is a first report on cryopreservation of bison semen without adding any exogenous protein in extender. In addition, artificial insemination of synchronized bison was effective and yielded pregnancies.

Below physiological temperatures, membrane destabilization due to lateral phase separation can result in impaired functions or cell death (Woelders, 1997). Cholesterol has a concentration dependent role in maintaining membrane stability (Nolan and Hammerstedt, 1997; Raffy and Teissié, 1999). Higher membrane cholesterol to phospholipid content increases lipid packing and reduces membrane disorganization associated with phase transitions (Boughter et al., 2016). Addition of cholesterol delivered by cyclodextrins to conventional TEYG extenders improved semen post-thaw characteristics in bull (Purdy and Graham, 2004b), ram (Mocé et al., 2010), boar (Lee et al., 2015) and wood bison (Hussain et al., 2013). However, cholesterol has never been used without egg yolk in cryopreservation of bovine semen. In the present study, egg yolk in extender was completely removed and sperm were treated with exogenous cholesterol. Cryopreservation of electroejaculated bison semen in CC-TG extender with the addition of 2 mg/mL CC yielded similar post-thaw motilities and velocities as the conventional TEYG extender. Our findings in the post-thaw characteristics of wood bison semen frozen with commercial egg yolk extender were comparable to those reported earlier (Hussain et al., 2013). Bison semen frozen in TEYG extender yielded higher post-thaw characteristics compared to the commercially available animal protein-free extender Andromed, which did not use CC (Lessard et al., 2009). This suggests that the membrane stabilizing effect of cholesterol alone is sufficient by itself to protect sperms during cryopreservation. Species with higher cholesterol:phospholipid ratio such as rabbits and humans, molar ratios 0.88, 0.99, respectively, are highly resistant against cold shock in comparison to species such as the boar and bull, molar ratios 0.35 and 0.45, respectively (Annabelle Darin-Bennett and White, 1977; Davis, 1981; Mocé and Vicente, 2009). Supplementation of cholesterol via CC (2 mg/mL) to bull sperm nearly doubled the cholesterol content of bull sperm (Purdy and Graham, 2004b), thus increasing the cholesterol:phospholipid

ratio similar to rabbit sperm. The cryosurvival of CC-treated (egg yolk-free) bison semen can likely be attributed to this increase in membrane stability and resistance against cold shock.

Glycerol increases membrane fluidity and permeability in sperm cells (Hammerstedt et al., 1992). Glycerolated portion of the semen extender was added at 22 °C or after initial cooling to 4 °C to CC-treated semen to determine the protective properties of exogenous cholesterol addition against cold shock without interference from glycerol. In the present study, glycerolization at 4 °C or 22 °C yielded similar post-thaw semen quality indicating that exogenous cholesterol alone was sufficient to protect the sperm during initial cooling. Our results agree with a previous study conducted in our lab where no differences in the post-thaw sperm quality were found between addition of glycerol at 37 °C or 4 °C using TEYG extender (Hussain et al., 2013). In other species, glycerol addition at 4 °C was beneficial for ram, boar and elephants (Almlid and Johnson, 1988; Gil et al., 2003; Kiso et al., 2012) These differences are likely species specific and are dependent on membrane resistance to osmotic changes. Addition of glycerol imposes osmotic stress upon the sperm membrane where the cells undergo rapid dehydration due to the initial hypotonic conditions. Bull sperm contain greater plasma membrane cholesterol content in comparison with ram and boar sperm (Annabelle Darin-Bennett and White, 1977). Therefore, they are more resistant against changes in osmolarity and thus cryosurvival was unaffected. Conversely, glycerol has the affinity to bind to the equatorial regions of cyclodextrins (Bastos et al., 1997; Moreira and Bastos, 2000), thereby it might interfere with the receptive centers of cyclodextrins. So, sperm must be exposed to CC first before adding glycerolated extender. Mechanism behind CC and glycerol interaction is still unclear and will require further investigation. Glycerolated portion of the extender can be added at room temperature (22 °C) for convenience.

The decline in sperm motilities and velocities after 2 h did not differ between TEYG and CC-TG extended semen. Maintaining sperm plasma membrane integrity is essential for its survival and fertilizing potential. Up to 50% of membrane cholesterol could be lost after cryopreservation which will result in reduced viability (Cerolini et al., 2001). The proportion of IPM-IACR in CC-TG semen did not differ with that in TEYG extender at 0 h and after 2 h of incubation period but maintained higher values at both time points. IPM-IACR were consistent with previous study in bison with TEYG extender yielding 32% at 0 h (Hussain et al., 2013). This suggested that cholesterol supplementation in TEYG extender was able to maintain greater membrane stability

after cryopreservation than egg yolk alone. Poor freezability of bison semen in Andromed have been suggested to be due to the lack of Andromed's ability to sequester bovine seminal plasma protein (BSPs) like factors (Lessard et al., 2009). Low density lipoproteins within egg yolk are suggested to prevent the negative effects of BSPs (Bergeron and Manjunath, 2006). If left unchecked, BSPs bind to cholesterol and phospholipids from the sperm plasma membrane and are removed together by capacitating factors such as heparin (Bailey, 2010), leading to poor sperm survival. Although CC-TG extender (without egg yolk) is not able to sequester BSP like factors, pre-treatment with CC appears to be sufficient to counteract the efflux of cholesterol by BSP like factors in bison semen.

The different kinds of frozen bison semen i.e. with or without egg yolk and dairy semen resulted in similar *in vitro* cleavage and blastocyst rates. Blastocyst formation following *in vitro* fertilization of cattle oocytes clearly indicated that bison semen with and without egg yolk have equal potential to undergo *in vitro* sperm capacitation, acrosome reaction and oocyte penetration. However, CC-TG extended semen did not yield any pregnancy.

In contrast, 9/21 (43%) pregnancy rate following FTAI in wood bison using TEYG extender was achieved. Our previous experiments did not indicate any difference in the post-thaw sperm characteristics and *in vitro* fertility between TEYG and CC-TG extended semen. We propose that the concentration of CC (2 mg/mL semen) may have negatively impacted normal sperm plasma membrane physiology. Cholesterol are incorporated into the sperm during maturation to maintain membrane stability within the female reproductive tract (Bailey, 2010). Removal of membrane cholesterol is one of the initial steps required for capacitation to occur (Bailey et al., 2000). Addition of exogenous cholesterol into media with mice sperm was able to prevent capacitation (Colazo et al., 2013). It was anticipated that exogenous cholesterol supplied by CC binding with bison sperm plasma membrane prevented capacitation from occurring. Small populations of sperm may have undergone cryocapacitation but was not enough to fertilize oocytes *in vivo*.

For acrosome reaction to occur, sperm are required to first undergo capacitation normally marked by cholesterol efflux and increase in intracellular Ca^{2+} (Bailey et al., 2000). Membrane damage accumulated from cryopreservation is often sufficient to induce capacitation-like changes and spontaneous acrosome reaction without an external stimulus. In the present study, we followed

distinct sperm populations depending on their plasma membrane integrity and acrosome status and determined that CC-TG semen had decreased levels of acrosome reaction in comparison to TEYG extender after incubation for 6 h. It was suggested that cholesterol:phospholipid ratio correlates with the time needed for capacitation to occur (Davis, 1981). This reinforced our previous notion that the supplementation of exogenous cholesterol increased membrane stability. In addition, there were no differences between cryopreservation induced acrosome reaction and LPC induced acrosome reaction. These results were supported by findings in Holstein bulls where no difference was found between cryo-induced and LPC-induced acrosome reaction at 6 h (Stout, 2012). Based on these results, we could assume that bison sperm needs a species-specific protocol for *in vitro* induction of acrosome reaction.

In summary, cryopreservation of bison semen in egg yolk-free extender with pretreatment using CC was successful and yielded acceptable post-thaw sperm characteristics. CC-TG extended bison semen kept their potential to *in vitro* fertilize an oocyte. However, they lost their ability to naturally fertilize an oocyte, suggesting a need for an optimization of the AI procedures in bison. Future work should focus on refining the current protocols using CC-TG cryopreservation method before its large scale implementation for commercial and conservation applications.

2.6. Acknowledgements

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CHAPTER 3: FERTILITY POTENTIAL OF BEEF AND BISON SEMEN FROZEN WITHOUT EXOGENOUS PROTEIN IN THE EXTENDER

3.1. Abstract

The objective of this study was to determine the appropriate concentration of cholesterol-cyclodextrin (CC) complex for semen to obtain fertility in bison and beef cows. Sperm motion characteristics, and plasma membrane and acrosome integrity were determined using computer assisted sperm analyzer and flow cytometer, respectively. Pregnancy rates using frozen semen were determined following fixed-time artificial insemination. In Experiment 1, semen was collected from Simmental bulls (n = 5) and pooled ejaculates were frozen in conventional Tris-egg yolk-glycerol extender (TEYG extender), or pretreated with 1 or 2 mg CC/mL and diluted in Tris-glycerol without egg yolk (CC-TG extender). Pre-freeze and post-thaw sperm motion characteristics were not different among treatment groups. Sperm in CC-TG extenders retained greater population of sperm with an intact plasma membrane and intact acrosome at all time points post-thaw. Frozen semen diluted in TEYG and 1 mg CC-TG extenders yielded greater pregnancy rates after a single fixed-time insemination compared to 2 mg CC-TG extender (50%, 49% vs. 13%, respectively; $P < 0.05$). In Experiment 2, an attempt was made to further reduce CC concentration for beef bull semen. Semen was collected from Simmental bulls (n = 3) and frozen separately in TEYG, 0.5 mg CC-TG or 1 mg CC-TG extenders. Single fixed-time insemination yielded pregnancy rates of 54%, 57% and 33% with semen in TEYG, 0.5 mg CC-TG semen and 1 mg CC-TG extenders, respectively. In Experiment 3, semen was collected from wood bison (n = 4), and pooled ejaculates were either directly diluted in TEYG extender, or pre-treated with 0.5 or 1 mg CC/mL semen before dilution in TG extender. Sperm in 1 mg CC-TG extender displayed comparable or better post-thaw motion characteristics than TEYG and 0.5 mg CC-TG extenders. After a single fixed-time insemination, pregnancy rates in bison were 55% and 36% with semen in TEYG and 1 mg CC-TG extenders, respectively. In conclusion, sperm motion, structural characteristics, and fertility were comparable using CC-TG vs TEYG extender. The lower concentration of CC (0.5 mg/mL semen) for pre-treatment of semen was associated with greatest fertility after fixed-time insemination in bovine and bison cows. Therefore, CC-TG extenders can be successfully used as a bio-secure alternative to cryopreserve beef and bison semen.

3.2. Introduction

Semen cryopreservation and artificial insemination are utilized extensively in the cattle industry and for conservation of threatened species. Lipoproteins, commonly of animal origin, such as egg yolk or milk, are necessary constituents in common semen extenders to protect sperm from initial cold shock during transition from room- to refrigerator-temperature (Pace and Graham, 1974). However, the use of animal products in semen extenders (i.e., egg yolk and milk) raises two main concerns: 1) they are of undefined composition, and 2) they are a potential source of microbial contamination, and thus pose a biosecurity risk for disease transmission (Bousseau et al., 1998). There is, therefore, a need to replace animal products such as egg yolk and milk in semen extenders with definable constituents that effectively prevent cold shock and minimize the potential of microbial contamination. Plant based extenders such as Andromed (Minitube USA, Delavan, Wisconsin, USA) have been used to cryopreserve bovine semen (Aires et al., 2003; Lima-Verde et al., 2017); however, the soybean lecithin-based extenders lack a well-defined composition which could affect consistency in semen production and research. Furthermore, in wild bovids, the presence of seminal plasma appears to be detrimental to sperm cryo-survival when frozen with Andromed (Herold et al., 2004; Lessard et al., 2009). Therefore, soy lecithin-based extenders are still under investigation and are not universally accepted (Layek et al., 2016b).

Cholesterol maintains membrane integrity by improving membrane stability and preventing phospholipids disorganization at lower temperatures (Boughter et al., 2016). Sperm with lower cholesterol content in their plasma membrane are less tolerant to cold shock compared to sperm with high cholesterol content (Annabelle Darin-Bennett and White, 1977; Drobnis et al., 1993). Loss of membrane cholesterol after cryopreservation induces premature capacitation and shorten sperm survival (Bailey, 2010). Cyclodextrins are cyclic oligosaccharides that act as carriers for non-polar structures, such as cholesterol, in aqueous environments, and have been used to deliver cholesterol into the cell membrane (Klein et al., 1995). Pretreatment with cholesterol-cyclodextrin (CC) complexes before dilution in egg yolk extender resulted in improved post-thaw quality in bovine and bison semen (Purdy and Graham, 2004a; Hussain et al., 2013)

While previous results demonstrated that bison semen frozen in CC-Tris-glycerol (CC-TG; egg yolk-free) extender yielded post-thaw sperm characteristics and *in vitro* fertilization (IVF) blastocyst rates comparable to that of conventional egg yolk extender, semen frozen in CC-TG

extender failed to produce any pregnancies in synchronized bison females following fixed-time artificial insemination (Yang, Chapter 2). Since cholesterol content of the sperm membrane differs among species (Parks and Lynch, 1992), we hypothesize that these differences account for the dichotomous fertility results between bovine and bison semen treated with CC (Yang, Chapter 2). The addition of exogenous cholesterol may have interfered with capacitation and subsequent acrosome reaction in bison sperm. Three experiments were conducted on bison and beef cattle to optimize the concentration of CC treatment using egg yolk-free extender for cryopreserving bison and bull semen, and to determine if the loss of fertility in CC-treated bison semen is associated with cryo-capacitation and premature acrosome reaction.

3.3. Materials & methods

3.3.1. Animals

Beef cattle at the Goodale Research Farm, and wood bison at the Native Hoofstock Center (University of Saskatchewan; 52° 10'N/106° 38'W) were used in this study. The respective species were maintained under uniform conditions of management and nutrition during the experimental periods. Wood bison were habituated to a special hydraulic chute system before experimentation to minimize stress. Animal procedures related to semen collection and fixed-time artificial insemination were approved by the Animal Care Committee, University of Saskatchewan. (Animal Use Protocol # 20100150).

3.3.2. Preparation of extenders and cholesterol-cyclodextrin complex

Conventional egg yolk extender

Conventional Tris-egg yolk-glycerol (TEYG) extender was prepared by adding glycerol (7%, v/v), egg yolk (20%, v/v), gentamycin sulfate (500 µg/mL), tylosin (100 µg/mL; Tylan Soluble, Elanco, Guelph, Ontario, Canada), and lincomycin-spectinomycin (300/600 µg/mL; Linco-Spectin, Pfizer Animal Health, Kirkland, Quebec, Canada) in Tris-citric acid (TCA) buffer (Tris base 3.03%, w/v, citric acid monohydrate 1.74%, w/v, and fructose 1.2%, w/v) in Milli-Q distilled water (pH 7.1). The mixture was centrifuged at 12,000x g for 15 min at 4 °C. The supernatant was recovered and stored at -20 °C. Extender was thawed in water bath (37 °C) before use.

Tris-glycerol (egg yolk-free) extender

Egg yolk-free extender, Tris-glycerol (TG, 2X concentration), contained glycerol (14%, v/v), gentamycin sulfate (1 mg/mL), tylosin (200 µg/mL), lincomycin-spectinomycin (600/1200 µg/mL) in TCA buffer. The extender was stored at -20 °C and warmed in a water bath (22 °C) before use. After incubation with CC, semen in TCA buffer was diluted (1:1) with TG (2X) to achieve the final concentrations of glycerol (7%, v/v), gentamycin sulfate (500 µg/mL), tylosin (100 µg/mL), and lincomycin-spectinomycin (300/600 µg/mL).

Preparation of cholesterol-cyclodextrin complex

Cholesterol-cyclodextrin complex was prepared as previously described (Purdy and Graham, 2004a). Solution A was prepared by dissolving cholesterol (200 mg, Cat. C8667, Sigma, St. Louis, Montreal) in 1 mL chloroform. Solution B was prepared by dissolving methyl β-cyclodextrins (1 gm, Cat. C4555 Sigma) in 2 mL methanol. Solution A (0.45 mL) was then added to Solution B (2 mL) and mixed until it became homogenous. The CC mixture was then poured into a glass Petri dish and dried under a gentle stream of nitrogen gas. The resulting crystals were further air-dried overnight in a desiccator and stored at 22 °C in a glass bottle. The working solution was prepared by dissolving CC in TCA buffer (50 mg CC/mL) at 22 °C on the day of the experiment.

3.3.3. General semen collection and evaluation

Semen was collected from beef and bison bulls by electroejaculation (Pulsator IV Auto Adjust; Lane Manufacturing Inc. Denver, CO, USA). Raw semen was kept at 32 °C after collection and transported to the laboratory within 2 h. Pre-freeze and post-thaw sperm total motility (TM), progressive motility (PM), average path velocity (VAP), curvilinear velocity (VCL) and straight-line velocity (VSL) were assessed by computer-assisted sperm analysis (CASA; Sperm Vision 3.0, Minitube Canada, Ingersoll, ON, Canada) following settings for bull semen (Anzar et al., 2011). A minimum of 350 sperm in 7 fields were evaluated by CASA.

Post-thaw plasma membrane and acrosome integrity were determined by flow cytometry using propidium iodide (PI; Invitrogen; stock 2.4 mM in water), and fluorescein isothiocyanate-peanut agglutinin (FITC-PNA; Sigma chemicals, St. Louis, MO; stock 1 mg/mL PBS) fluorescent markers, as previously described (Anzar et al., 2011; Hussain et al., 2013; Yang, Chapter 2). In

brief, washed semen was diluted to $1x10^6$ sperm/mL with sp-TALP (100 mM NaCl, 3.1 mM KCl, 0.3 mM NaH_2PO_4, 0.4 mM $MgCl_2$, 10 mM HEPES, 0.4 mM EDTA, 21.6 mM lactate, 2 mM $CaCl_2$, 1 mM pyruvate, 25 mM $NaHCO_3$, 6 mg/mL BSA). The semen samples were then incubated at 39 °C and 5% CO_2 for up 6 h. At 0, 4, or 6 h of incubation, aliquots of the semen samples were extracted and incubated with 5 µL PI and 1 µL FITC-PNA at 22 °C in the dark for 20 min. Sperm were fixed by adding 10 µL of 10% formaldehyde after incubation before flow cytometry.

3.3.4. Experiment 1. Effect of CC concentration on bovine sperm motion characteristics and fertility.

Semen processing

Semen was collected from Simmental bulls (n = 5 bulls, 5 ejaculates per bull; 1 ejaculate per week) during the months of May and June. Ejaculates with sperm total motility of ≥60% and sperm concentration of $\geq 200x10^6$/mL were pooled and divided into 3 aliquots. Semen aliquot 1 was diluted to $50x10^6$ sperm/mL with TEYG extender at 37 °C and kept at 22 °C for 35 min. Semen aliquots 2 and 3 were initially diluted to $100x10^6$ sperm/mL with TCA buffer at 37 °C and cooled to 22 °C over a period of 20 min, and then incubated with 1 and 2 mg CC/mL semen, respectively, at 22 °C for 15 min (hereafter referred to as 1 and 2 mg CC-TG, respectively). After incubation with CC complex, semen was further diluted (1:1) with egg yolk-free TG extender (2X) at 22 °C. The semen aliquots (TEYG, 1 mg CC-TG, and 2 mg CC-TG) were then cooled to 4 °C in a walk-in cooler for 90 to 120 min, and packaged in 0.5 mL French straws. Semen was then frozen in a programmable cell freezer (ICE-CUBE 14-S, Sy-Lab Version 1.30, Gerate GmbH, Neupurkdersdof, Austria): -3 °C/min from 4 °C to -10 °C, and -40 °C/min from -10 °C to -80 °C; the semen straws were then plunged into liquid nitrogen (Hussain et al., 2011). Two straws were thawed at 37 °C for at least 1 min, pooled, and analyzed by CASA and flow cytometer. Flow cytometry analysis revealed four different sperm populations based on their plasma membrane and acrosome integrities; i.e., intact plasma membrane and intact acrosome (IPM-IACR), intact plasma membrane and reacted acrosome (IPM-RACR), compromised plasma membrane and intact acrosome (CPM-IACR) and compromised plasma membrane and reacted acrosome (CPM-RACR) (Anzar et al., 2011).

Fixed-time artificial insemination in beef cows

A 2 x 3 factorial design was used to examine the effects of ovarian synchronization protocols (progesterone vs non-steroidal) and semen extenders (TEYG, 1 mg CC-TG or 2 mg CC-TG) on pregnancy rate in cows. Multiparous Hereford-cross beef cows (n = 120), ≥45 days post-partum with normal uterine involution and a corpus luteum (confirmed by transrectal ultrasonography) were used during the month of June. As part of another study, cows at random and unknown stages of the estrous cycle were assigned randomly to either a progesterone-based synchronization protocol (n = 58) or a nonsteroidal-based protocol (n = 62; Fig. 3.1). In the progesterone-based group, an intravaginal progesterone-releasing device (Cuemate, Vetoquinol, Lavaltrie, Quebec Canada) was inserted on Day 0 and removed on Day 5. Cows were given a luteolytic dose of PGF2α (500 µg cloprostenol *im;* Estrumate, Merk, Kirkland, Québec, Canada) at the time of device removal, and were given GnRH (100 µg gonadorelin *im*, Fertiline, Vetoquinol, Lavaltrie, Québec, Canada) and inseminated 72 h later. In the nonsteroidal-based group, an intravaginal device was inserted on Day 0 and removed on Day 4. Cows were given a luteolytic dose of PGF2α at the time of device removal, and were given GnRH and inseminated 48 h later. Cows were inseminated with a single dose of semen extended and frozen in either TEYG, 1 mg CC-TG or 2 mg CC-TG. Ovarian activity for a subset of cows in each synchronization group (n = 8) was examined every 8 h (beginning 24 h after vaginal device removal) until ovulation was detected. Pregnancy was diagnosed 28 days after insemination by transrectal ultrasonography.

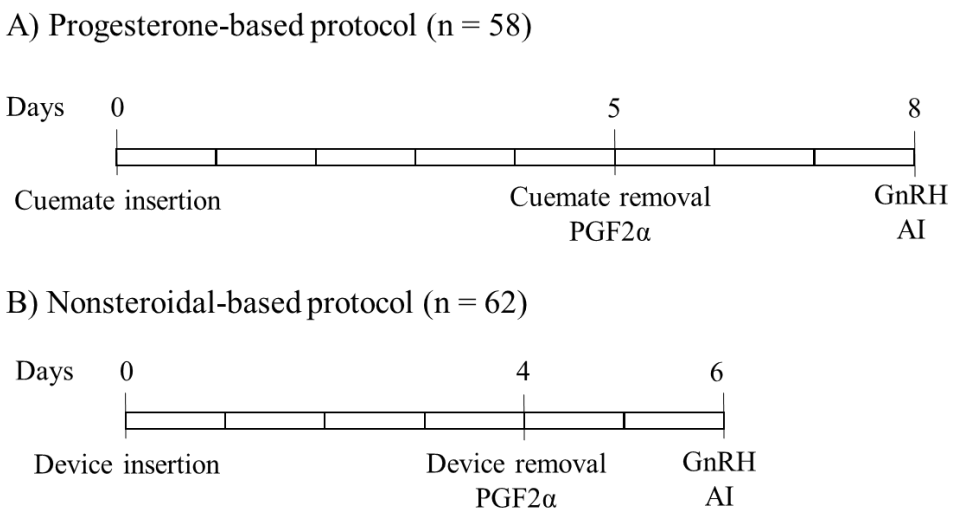


Fig. 3.1. Ovarian synchronization protocols for fixed-time artificial insemination in multiparous Hereford cross beef cows (Experiment 1).

3.3.5. Experiment 2. Effect of low-dose cholesterol-cyclodextrin complex on sperm motion characteristics and pregnancy rates in beef cows

Semen processing

Semen was collected from Simmental bulls (n = 3 bulls, 5 ejaculates per bull; 1 ejaculate per week) during the months of May and June. Ejaculates with sperm total motility of ≥60% and sperm concentration of ≥200x10^6/mL were processed by individual bull and further divided into 3 aliquots. Semen aliquot 1 was diluted to 50x10^6 sperm/mL with TEYG extender at 37 °C and kept at 22 °C for 35 min. Semen aliquots 2 and 3 were initially diluted to 100x10^6 sperm/mL with TCA buffer at 37 °C and cooled to 22 °C over a period of 20 min, and then incubated with 0.5 and 1 mg CC/mL semen, respectively, at 22 °C for 15 min (hereafter referred to as 0.5 and 1 mg CC-TG, respectively). After incubation with CC complex, semen was diluted (1:1) with egg yolk-free TG extender (2X) at 22 °C to reach final concentration of 50x10^6 sperm/mL. The diluted semen was packaged in 0.5 mL French straws, cooled and frozen, as described in Experiment 1. Pre-freeze and post-thaw semen characteristics were analyzed by CASA. Sperm motion characteristics were compared among extenders within each bull.

Fixed time artificial insemination in beef cows

Multiparous Hereford-cross beef cows (n = 147), ≥45 days post-partum with normal uterine involution and a corpus luteum (confirmed by transrectal ultrasonography) were used during the month of June. At random and unknown stages of the estrous cycle, an intravaginal progesterone-releasing device (PRID, Vetoquinol, Lavaltrie, Québec, Canada) was inserted (Day 0), and removed on Day 5. A luteolytic dose of PGF2α (500 ug cloprostenol *im*) was given on the day of device removal and on Day 8, cows were treated with GnRH and inseminated with semen extended and frozen in either TEYG, or 0.5 mg CC-TG, or 1 mg CC-TG. Bull C was excluded since its semen frozen in CC-TG extenders did not meet the minimum motility value of the control group. Instead, only Bull A and B were used to examine the effect of semen extender on pregnancy rates given that they had similar post-thaw sperm characteristics. Bulls and semen treatments were assigned randomly among cows. Ovarian ultrasonography was done on Day 8 (time of insemination) to record the size and location of the dominant follicle, and ovulation was confirmed on Day 13 by the presence of corpus luteum in the ovary that had the dominant follicle. Pregnancy was diagnosed 30 days after insemination by ultrasonography.

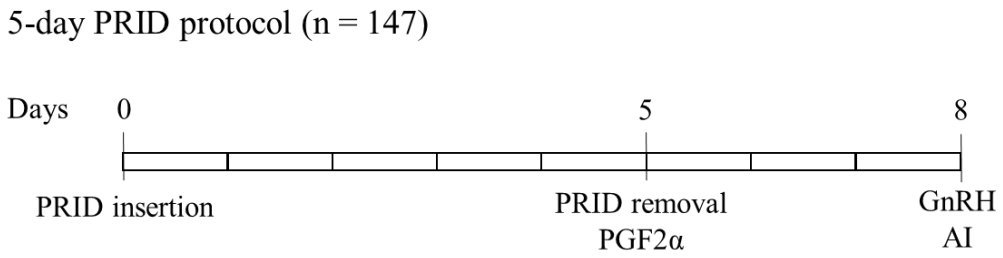


Fig. 3.2. Ovarian synchronization protocol for fixed-time artificial insemination in multiparous Hereford-cross beef cows (Experiment 2).

3.3.6. Experiment 3: Effect of CC concentration on bison sperm motion characteristics and fertility.

Semen processing

Semen was collected from bison bulls (n = 4, up to 5 ejaculates per bull) during the breeding season (November). Ejaculates with sperm total motility of ≥60% and sperm concentration of ≥200x10^6/mL were pooled and divided into 3 aliquots. Semen aliquot 1 was diluted with TEYG extender to the final concentration of 50x10^6 sperm/mL. Semen aliquots 2 and 3 were initially treated with 0.5 or 1 mg CC/mL semen respectively, at 22 °C for 15 min and then diluted in egg yolk-free TG extender (2X). Semen was then cooled to 4 °C, packaged in 0.5 mL French straws and frozen in a programmable cell freezer as described in Experiment 1. Pre-freeze and post-thaw semen characteristics were analyzed by CASA.

Fixed-time artificial insemination in wood bison

A 2 x 2 factorial design was conducted during the ovulatory season (November to December) to examine the effects of ovarian synchronization protocol and semen extender on pregnancy rate in bison. Synchronization protocols for bison in a previous study (Yang, Chapter 2) were modified based on ovarian synchrony results (Fig. 3.3). At random and unknown stages of the estrous cycle, bison were assigned randomly to either a follicular ablation group (n = 12) or estradiol+progesterone group (n = 11). Follicular ablation was accomplished by transvaginal ultrasound-guided aspiration of ovarian follicles ≥5 mm using convex array ultrasound probe (Aloka SSD 900; Tokyo, Japan). In the estradiol+progesterone group, bison females were given 2.5 mg E-17ß and 50 mg progesterone in canola oil *im*. The day of ablation or estradiol+progesterone treatment was considered Day 0. A progesterone-releasing intravaginal device (PRID, Vetoquinol, Lavaltrie, Québec) was inserted on Day 0 for both protocols. On Days 6 and 8 for the ablation and estradiol+progesterone groups, respectively, the PRID was removed and bison were treated with a luteolytic dose of PGF2α (500 µg cloprostenol *im*). Human chorionic gonadotropin (hCG, 2500 IU Chorulon, Merck Animal Health, Kirkland, Québec, Canada) was administered *im* 72 h after PGF2α treatment to induce ovulation (Palomino et al., 2015, 2014a). Bison were inseminated 12 and 24 h post-hCG with semen extended and frozen in TEYG or 1 mg CC-TG extender (Fig. 3.3). Semen treated with 0.5 mg CC were excluded because it did not meet the minimum motility value of the control group. Ovulation was confirmed by serial

ultrasonography, every 12 h from the initial insemination for up to 4 days. Pregnancy was diagnosed 40 days after insemination by ultrasonography.

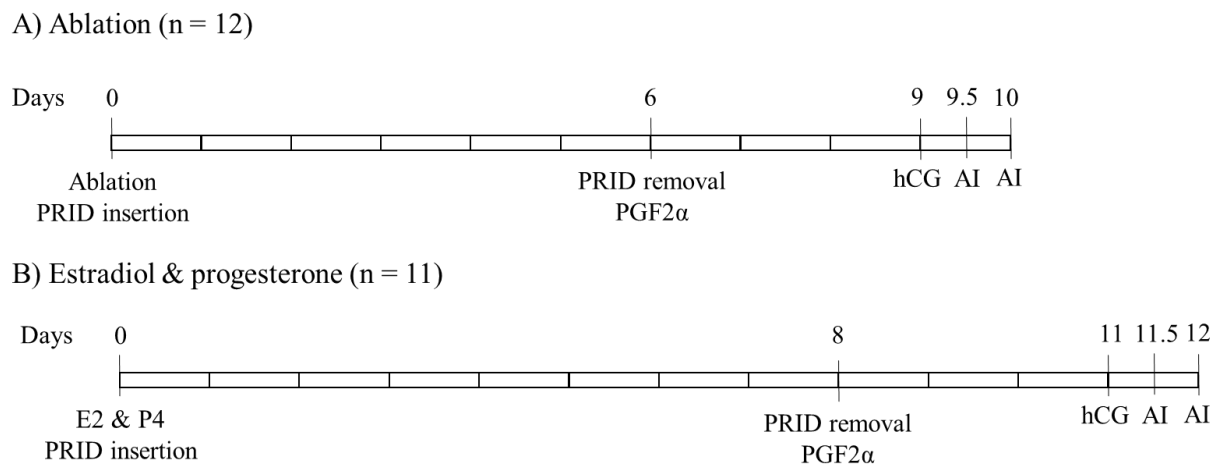


Fig. 3.3. Ovarian synchronization protocols for fixed-time artificial insemination in wood bison (Experiment 3).

3.3.7. Statistical analysis

Values are expressed as mean ± standard error of the mean (SEM) unless otherwise stated. Sperm motion characteristics, and plasma membrane and acrosome integrities were compared among groups using repeated measures analysis of variance. The decline in sperm parameters from pre-freeze to post-thaw stages was expressed as Δ. Pregnancy rates among groups were compared by binomial generalized linear mixed model analysis of variance. If $P < 0.05$, the means were separated with Tukey's test. Data analyses were performed using the R Project for Statistical Computing Package (R version 3.3.1, R Foundation for Statistical Computing, Vienna, Austria).

3.4. Results

3.4.1. Experiment 1: Effect of CC concentration on bovine sperm motion characteristics and fertility.

Post-thaw sperm total motility, progressive motility, VAP, VCL and VSL did not differ among TEYG, 1 mg CC-TG and 2 mg CC-TG extenders (Table 3.1). Sperm total and progressive

motilities were lower ($P < 0.05$) in post-thaw than pre-freeze stage, across all extenders. Likewise, post-thaw sperm average path velocity in 1 mg and 2 mg CC-TG semen and curvilinear velocity in 2 mg CC-TG semen were lower ($P < 0.05$) than the pre-freeze stage. Extender had no effect on decline (Δ) in sperm motion characteristics from pre-freeze to post-thaw stage.

Table 3.1

Sperm motion characteristics (mean ± SEM, n = 5 pooled ejaculates) in beef semen diluted and frozen in conventional Tris-egg yolk-glycerol extender (TEYG), or pretreated with 0.5 or 1 mg cholesterol-cyclodextrin/mL of semen and diluted in Tris-glycerol without egg yolk (CC-TG). The decline in sperm motion characteristics from pre-freeze to post-thaw stages is expressed as *Δ*.

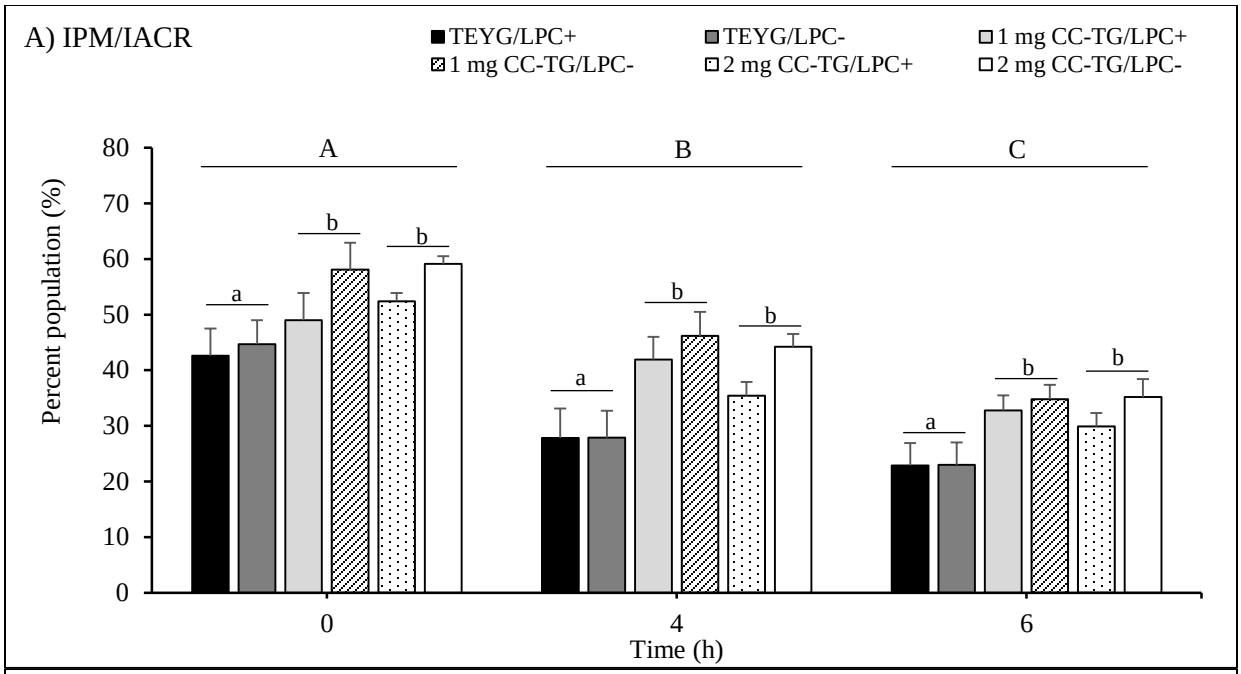
Sperm characteristics		Semen extender*		
		TEYG	1 mg CC-TG	2 mg CC-TG
Total motility (%)	Pre-freeze	85±3.5 [x]	70±3.6 [x]	73±5.2 [x]
	Post-thaw	58±6.2 [y]	51±3.9 [y]	49±6.7 [y]
	Δ	*28±4.5*	*19±1.9*	*24±3.2*
Progressive motility (%)	Pre-freeze	79±4.3 [x]	63±4.4 [x]	67±5.6 [x]
	Post-thaw	50±6.7 [y]	46±4.5 [y]	44±7.4 [y]
	Δ	*29±4.6*	*18±1.4*	*23±2.9*
Velocity - average path (VAP, µm/s)	Pre-freeze	65±3.7	70±3.3 [x]	72±6.0 [x]
	Post-thaw	64±1.6	61±3.5 [y]	60±4.0 [y]
	Δ	*1±3.0*	*8±3.2*	*12±3.3*
Velocity - curvilinear (VCL, µm/s)	Pre-freeze	121±10.2	133±7.4	143±12.6 [x]
	Post-thaw	118±6.2	118±9.1	119±9.9 [y]
	Δ	*3.3±6.9*	*14±8.1*	*24±9.1*
Velocity - straight line (VSL, µm/s)	Pre-freeze	46±1.1	48±1.6	42±2
	Post-thaw	48±0.8	45±2.0	43±2.1
	Δ	*-2±1.7*	*3±1.6*	*-1±1.8*

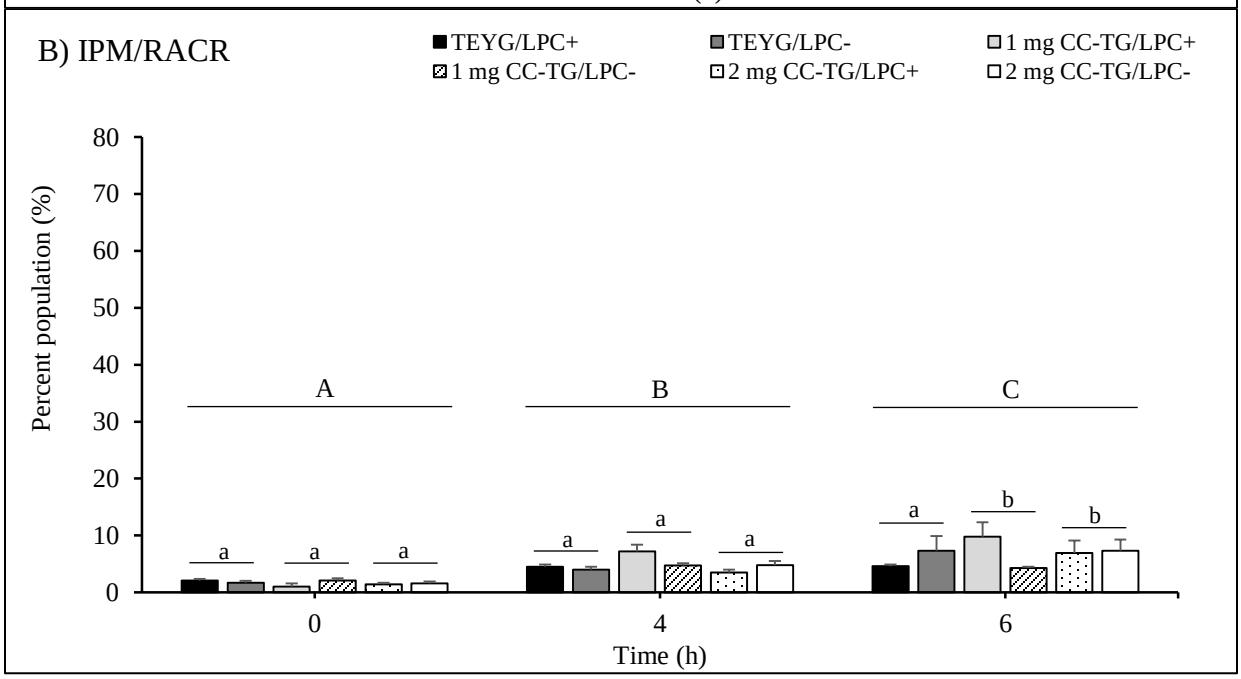
[xy]Within columns, pre-freeze and post-thaw values are different ($P < 0.05$).

*No significant differences among semen extenders for any characteristic.

There was a significant effect of time on IPM/IACR, IPM/RACR, and CPM/RACR sperm population at 4 and 6 h of incubation at 39 °C (Fig. 3.4). However, CPM/IACR sperm population did not change over time (Fig. 3.4C). At 0 h, semen in TEYG extender had significantly lower IPM/IACR population as compared to 1 mg or 2 mg CC-TG extender. After incubation for 4 and 6 h, IPM-IACR sperm population remained low with TEYG extender (Fig. 3.4A).

Correspondingly, at 0 h, semen in TEYG extender had significantly greater CPM/RACR sperm population as compared to 1 mg CC-TG extender. After incubation for 4 and 6 h, both 1 mg CC-TG extender had significantly lower CPM/RACR sperm population as compared to TEYG extender (Fig. 3.4D). No significant effect of LPC treatment in all sperm populations at each time point was observed.


A) IPM/IACR
TEYG/LPC+
TEYG/LPC-
1 mg CC-TG/LPC+
1 mg CC-TG/LPC-
2 mg CC-TG/LPC+
2 mg CC-TG/LPC-
Percent population (%)
Time (h)
A
B
C
a
b


B) IPM/RACR
TEYG/LPC+
TEYG/LPC-
1 mg CC-TG/LPC+
1 mg CC-TG/LPC-
2 mg CC-TG/LPC+
2 mg CC-TG/LPC-
Percent population (%)
Time (h)
A
B
C
a
b

(Continued)

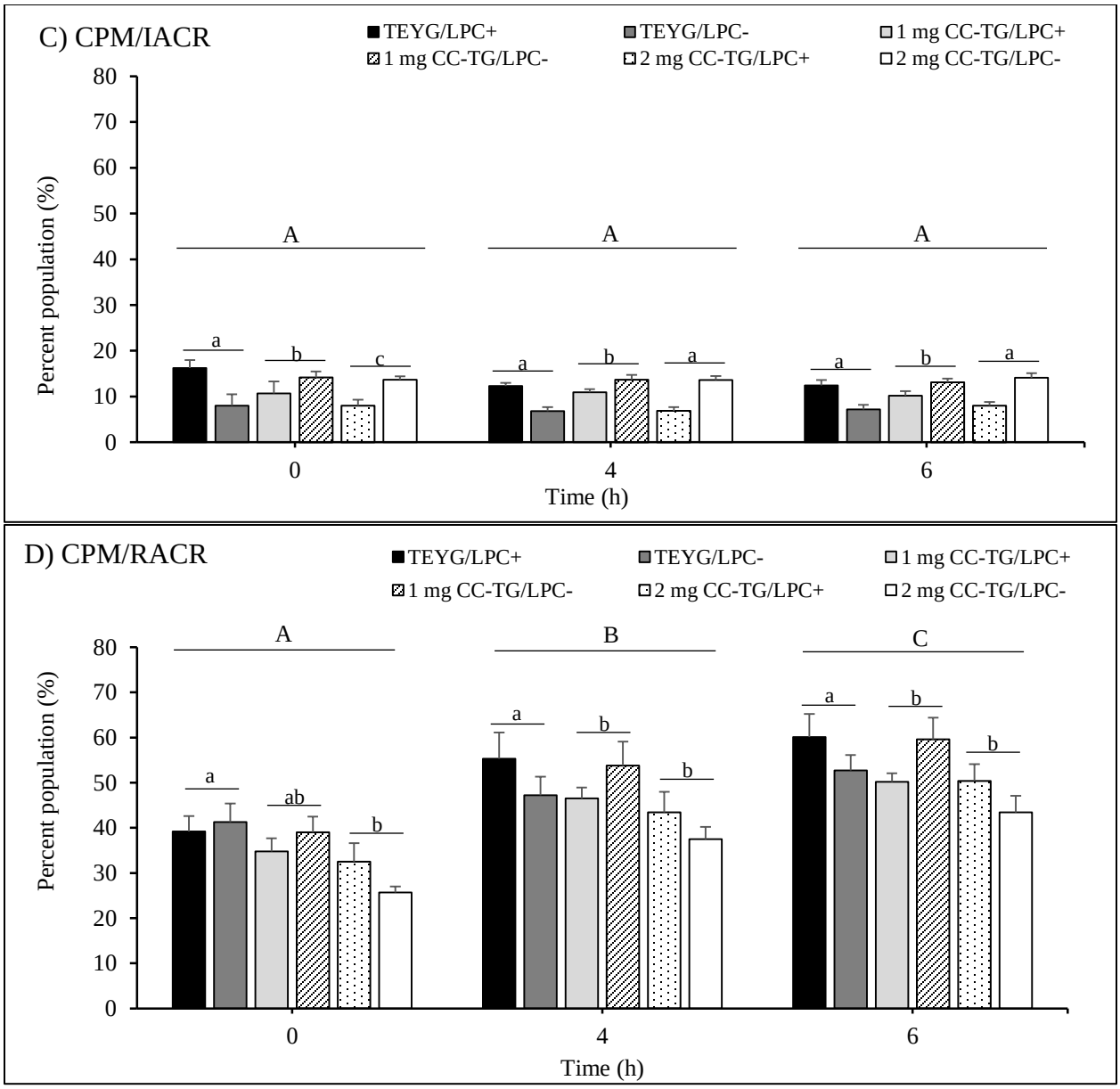


Fig. 3.4. Sperm plasma membrane and acrosome integrities in TEYG, 1 mg CC-TG and 2mg CC-TG extenders, after incubation with (+) or without (-) LPC at 0, 4, and 6 h (n = 5 frozen semen samples). Letters A-C denote significant difference among time and letters a-c denote significant difference among extenders.

Abbreviations: IPM/IACR, sperm with intact plasma membrane and intact acrosome (A, PI^-/FITC-PNA^-); IPM/RACR, sperm with intact plasma membrane and reacted acrosome (B, PI^-/FITC-PNA^+); CPM/IACR, sperm with compromised plasma membrane and intact acrosome (C, PI^+/FITC-PNA^-); CPM/RACR, sperm with compromised plasma membrane and reacted acrosome (D, PI^+/FITC-PNA^-).

Data on pregnancy rate in different semen extenders and synchronization protocols are presented in Table 3.2. Of the two synchronization protocols used, progesterone based protocol yielded higher pregnancy rate in comparison to the non-steroidal based protocol (50% vs 26%, P < 0.05). Within non-steroidal based protocol, semen in 2 mg CC-TG extender yielded no pregnancy. There was no interaction between synchronization protocols and semen extenders on pregnancy rate; therefore, the data were combined over synchronization protocol to examine the effect of semen extenders on pregnancy rates. Total pregnancy rates were 20/40 (50%), 20/41 (49%) and 5/39 (13%) for TEYG, 1 mg CC-TG and 2 mg CC-TG extenders, respectively. Semen in 2 mg CC-TG extender yielded significantly lower pregnancy rate as compared to TEYG and 1 mg CC-TG extenders (P < 0.05).

Table 3.2.

Pregnancy rates in synchronized beef cows (number pregnant/number that ovulated) after fixed-time insemination with semen diluted and frozen in conventional Tris-egg yolk-glycerol extender (TEYG), or pretreated with 1 or 2 mg cholesterol-cyclodextrin/mL of semen and diluted in Tris-glycerol without egg yolk (CC-TG).

Semen extender	Synchronization protocol		Total
	Progesterone based	Non-steroidal based	
TEYG	13/20 (65%)	7/20 (35%)	$20/40^{x}$ (50%)
1 mg CC-TG	11/19 (58%)	9/22 (41%)	$20/41^{x}$ (49%)
2 mg CC-TG	5/19 (26%)	0/20 (0%)	$5/39^{y}$ (13%)
Total	$29/58^{a}$ (50%)	$16/62^{b}$ (26%)	45/120 (38%)

[ab]Within rows, pregnancy rates with different superscripts are different (P < 0.05).

[xy]Within columns, pregnancy rates with different superscripts are different (P < 0.05).

3.4.2. Experiment 2. Effect of low-dose cholesterol-cyclodextrin complex on sperm motion characteristics and pregnancy rates in beef cows

Data on post-thaw sperm characteristics of semen extenders within individual bulls are presented in Table 3.3. For Bull A, there was no difference among semen extenders in total and

progressive motilities, and average path velocity. For Bull B, total and progressive motilities, and average path velocity were lower in 0.5 mg CC-TG extender as compared to TEYG and 1 mg CC-TG extenders. For Bull C, total and progressive motilities, and average path velocity differed among semen extenders with TEYG yielding the highest values and 0.5 mg CC-TG yielding the lowest values. Both Bull A and Bull B had greater curvilinear velocity in 0.5 and 1 mg CC-TG extenders as compared to TEYG extender.

Table 3.3.

Post-thaw sperm motion characteristics (mean ± SEM) of ejaculates from three bulls diluted and frozen in conventional Tris-egg yolk-glycerol extender (TEYG), or pretreated with 0.5 or 1 mg cholesterol-cyclodextrin/mL of semen and diluted in Tris-glycerol without egg yolk (CC-TG).

Bull*	Semen extender	Sperm characteristics				
		Total motility (%)	Progressive motility (%)	Velocity - average path (VAP, µm/s)	Velocity - curvilinear (VCL, µm/s)	Velocity - straight-line (VSL, µm/s)
A	TEYG	70±2.7	64±3.1	57±2.4	99±5.9^{x}	44±1.8
	0.5 mg CC-TG	68±3.3	62±3.8	61±0.5	116±1.0^{y}	46±1.1
	1 mg CC-TG	66±4.7	59±5.2	60±1.8	112±4.2^{y}	44±1.7
	Mean	*68*±2.0	*62±2.3*	*59±1.1*	*109±2.9*	*45±0.9*
B	TEYG	58±5.3^{x}	52±5.3^{x}	56±1.2^{x}	102±2.9^{x}	43±1.2
	0.5 mg CC-TG	47±5.9^{y}	41±5.7^{y}	61±1.1^{y}	121±2.6^{y}	43±0.9
	1 mg CC-TG	63±3.2^{x}	57±2.8^{x}	62±0.7^{y}	124±2.0^{y}	42±0.9
	Mean	*56±3.2*	*50±3.1*	*60±0.9*	*116±2.9*	*43±0.5*
C	TEYG	52±4.0^{x}	45.0±3.4^{x}	56±2.8	104±6.9	43±2.1
	0.5 mg CC-TG	21±5.9^{y}	16±5.1^{y}	56±3.5	109±7.8	40±2.5
	1 mg CC-TG	29±6.5^{z}	24±5.9^{z}	57±3.6	114±9.5	41±1.9
	Mean	*34±4.9*	*29±4.5*	*56±1.8*	*109±4.4*	*41±1.2*

*Bull A, n = 5 ejaculates; Bull B, n = 5 ejaculates; Bull C, n = 4 ejaculates.

^{xyz}Within columns, values with different superscripts are different for a given bull ($P < 0.05$).

Data from all three bulls were combined to compare sperm motion characteristics among semen extenders (Table 3.4). There was a significant decline in total and progressive motilities

and curvilinear velocity from the pre-freeze to post-thaw stages across all semen extenders. TEYG semen had greater total and progressive motilities at the pre-freeze stage as compared to 0.5 mg CC-TG semen. However, semen in TEYG extender experienced greater decline in total and progressive motilities from pre-freeze to post-thaw stages as compared to 0.5 mg CC-TG extender. There was no difference for motilities between TEYG and 1 mg CC-TG extenders. Average path velocity at all stages of freezing and decline in average path velocity was greater with 1 mg CC-TG as compared to TEYG extender. In addition, both 0.5 and 1 mg CC-TG extenders showed greater curvilinear velocity as compared to TEYG at pre-freeze and post-thaw stages. Straight line velocity did not differ among semen extenders.

Table 3.4.

Sperm motion characteristics (mean ± SEM, n = 14 pooled ejaculates) in beef semen diluted and frozen in conventional Tris-egg yolk-glycerol extender (TEYG), or pretreated with 0.5 or 1 mg cholesterol-cyclodextrin/mL of semen and diluted in Tris-glycerol without egg yolk (CC-TG). The decline in sperm motion characteristics from pre-freeze to post-thaw stages is expressed as *Δ*.

Sperm characteristics		Semen extender			P-value
		TEYG	0.5 mg CC-TG	1 mg CC-TG	
Total motility (%)	Pre-freeze	87±1.5^{a}	66±5.7^{b}	76±4.7^{ab}	< 0.05
	Post-thaw	61±3.0^{a}*	47±5.9^{b}*	54±5.1^{ab}*	
	Δ	27±2.2^{a}	18±1.7^{b}	22±2.4^{ab}	*< 0.05*
Progressive motility (%)	Pre-freeze	83±1.5^{a}	60±5.9^{b}	71±5.0^{ab}	< 0.05
	Post-thaw	54±3.0*	42±5.7*	49±5.0*	
	Δ	*28±2.2^{a}*	*18±1.6^{b}*	*23±2.3^{ab}*	*< 0.05*
Velocity - average path (VAP, µm/s)	Pre-freeze	65±0.8^{a}	69±1.4^{a}	74±1.2^{b}	< 0.05
	Post-thaw	56±1.2^{a}	60±1.2^{ab}	60±1.3^{b}	
	Δ	9±0.8^{a}	9±1.7^{a}	14±1.5^{b}	*< 0.05*
Velocity - curvilinear (VCL, µm/s)	Pre-freeze	*125±2.3^{a}*	*139±3.2^{b}*	*151±2.9^{c}*	*< 0.05*
	Post-thaw	102±2.9^{a}*	116±2.5^{b}*	117±3.3^{b}*	
	Δ	23±1.7	23±4.1	34±3.9	*NS*
Velocity - straight line (VSL, µm/s)	Pre-freeze	44±0.8	44±1.6	46±1.3	< 0.05
	Post-thaw	43.0.9	43±1.0	43±0.9*	
	Δ	1±0.7	1±1.3	4±1.0	*NS*

^{ab}Within rows, values with different superscripts are different. P-value belongs to repeated measure analysis of variance showing the overall effect of treatments on sperm characteristics.

*Within columns, pre-freeze and post-thaw values are different (P < 0.05).

Data on pregnancy rate in different semen extenders and bulls are presented in Table 3.5. Out of 147 synchronized cows, 132 cows were confirmed to have a luteum on day 13 (90% ovulation rate). Pregnancy rates in cows ovulated were 25/46 (54%), 26/46 (57%) and 13/40 (33%) in TEYG, 0.5 mg CC-TG, and 1 mg CC-TG extenders, respectively. Overall, pregnancy rate with 1 mg CC-TG extender was significantly lower ($P < 0.05$) than both TEYG and 0.5 CC-TG extenders. The overall pregnancy rate did not differ between bulls ($P = 0.87$). Difference in pregnancy rate among bulls within semen extender was observed. Bull A and B yielded similar pregnancy rate with TEYG and 0.5 mg CC-TG extender. However, Bull A yielded lower pregnancy rate compared to Bull B (15% vs 50%, respectively; $P < 0.05$) using 1 mg CC-TG extender.

Table 3.5.

Pregnancy rates in synchronized beef cows (number pregnant/number that ovulated) after fixed-time insemination with semen frozen in conventional Tris-egg yolk-glycerol extender (TEYG), or pretreated with 0.5 or 1 mg cholesterol-cyclodextrin/mL of semen and diluted in Tris-glycerol without egg yolk (CC-TG).

Semen extender	Bull A	Bull B	Total
TEYG	15/24 (63%)	10/22 (45%)	$25/46^{x}$ (54%)
0.5 mg CC-TG	15/25 (60%)	11/21 (52%)	$26/46^{x}$ (56%)
1 mg CC-TG	$3/20^{a}$ (15%)	$10/20^{b}$ (50%)	$13/40^{y}$ (33%)
Total	33/69 (48%)	31/63 (49%)	64/132 (48%)

[ab]Within rows, values with different superscripts are different.

[xy]Within columns, values with different superscripts are different ($P < 0.05$).

3.4.3. Experiment 3: Effect of CC concentration on bison sperm motion characteristics and fertility.

Pre-freeze sperm total and progressive motilities did not differ among TEYG, 0.5 mg CC-TG and 1 mg CC-TG extenders. However, post-thaw total and progressive motilities were higher with semen in 1 mg CC-TG than both TEYG and 0.5 mg CC-TG extenders (Table 3.6). Sperm

average path, curvilinear and straight line velocities were lower ($P < 0.05$) in TEYG extender as compared to both 0.5 mg CC-TG and 1 mg CC-TG extenders at both pre-freeze and post-thaw stages. Sperm total and progressive motilities, and average path velocity were lower ($P < 0.05$) at the post-thaw stage as compared to the pre-freeze stage, in all extenders. In addition, semen in 1 mg and 2 mg CC-TG extenders had lower curvilinear velocity as compared to TEYG extender at the post-thaw stage as compared to the pre-freeze stage. Semen in TEYG extender experienced a greater decline in total and progressive motility as compared to 1 mg CC-TG extender from the pre-freeze to post-thaw stage ($P < 0.05$). Extenders had no effect on decline in sperm velocities from pre-freeze to post-thaw stage.

Table 3.6.

Sperm motion characteristics (mean ± SEM) in bison semen (n = 5 bison, pooled ejaculates) diluted and frozen in conventional Tris-egg yolk-glycerol extender (TEYG), or pretreated with 0.5 or 1 mg cholesterol-cyclodextrin/mL of semen and diluted in Tris-glycerol without egg yolk (CC-TG). The decline in sperm motion characteristics from pre-freeze to post-thaw stages is expressed as *Δ*.

Sperm characteristics		Semen extender			
		TEYG	0.5 mg CC-TG	1 mg CC-TG	P-value
Total motility (%)	Pre-freeze	88±1.6	80±2.7	85±1.6	< 0.05
	Post-thaw	50±2.6^{a}*	46±4.1^{a}*	59±2.9^{b}*	
	Δ	*38±3.0^{a}*	*35±2.6^{ab}*	*26±2.0^{b}*	*< 0.05*
Progressive motility (%)	Pre-freeze	82±2.0	77±3.0	82±1.9	< 0.05
	Post-thaw	39±2.9^{a}*	40±4.4^{a}*	54±2.7^{b}*	
	Δ	*43±3.3^{a}*	*36±2.8^{ab}*	*28±1.6^{b}*	*< 0.05*
Velocity - average path (VAP, µm/s)	Pre-freeze	55±0.9^{a}	78±2.0^{b}	77±3.6^{b}	< 0.05
	Post-thaw	50±1.8^{a}*	67±3.3^{b}*	67±2.7^{b}*	
	Δ	*5±1.7*	*11±2.6*	*10±3.2*	*NS*
Velocity - curvilinear (VCL, µm/s)	Pre-freeze	92±1.9^{a}	158±5.3^{b}	156±9.3^{b}	< 0.05
	Post-thaw	80±1.9^{a}	129±6.9^{b}*	130±5.6^{b}*	
	Δ	*12±2.7*	*29±5.6*	*25±8.4*	*NS*
Velocity - straight line (VSL, µm/s)	Pre-freeze	45±0.9^{a}	51±0.7^{b}	52±1.9^{b}	< 0.05
	Post-thaw	42±1.8^{a}	50±2.9^{b}	51±2.4^{b}	
	Δ	*2±1.4*	*1±2.2*	*1±1.7*	*NS*

^{ab}Within rows, values with different superscripts are different among extenders. P-value belongs to repeated measure analysis of variance showing the overall effect of treatments on sperm characteristics.

Asterisks (*) represent differences between pre-freeze and post-thaw stage within each semen characteristics.

With better post-thaw sperm characteristics, 1 mg CC-TG extender was selected over 0.5 mg CC-TG extender for artificial insemination and compared with TEYG extender, due to limited number of animals available. Data on pregnancy rate in different semen extenders and synchronization protocols are presented in Table 3.7. Ovarian synchronization protocol did not have an effect on pregnancy rates in bison. In addition, no effect of interaction between synchronization protocols and semen extenders was detected in the statistical analysis. Therefore, the data on pregnancy rates were pooled over synchronization protocols to analyse the effect of semen extender on pregnancy rate. One inseminated animal did not ovulate and therefore was excluded from the analysis. Pregnancy rates were 6/11 (55%) and 4/11 (36%) for TEYG and 1 mg CC-TG extenders, respectively (NS).

Table 3.7.

Pregnancy rates in synchronized bison (number pregnant/number that ovulated) after fixed-time insemination with semen diluted and frozen in conventional Tris-egg yolk-glycerol extender (TEYG), or pretreated with cholesterol-cyclodextrin and diluted in Tris-glycerol without egg yolk (CC-TG).

Semen extender	Synchronization protocol		Total
	Ablation	E2 & P4	
TEYG	3/5 (60%)	3/6 (50%)	6/11 (55%)
1 mg CC-TG	3/7 (43%)	1/4 (25%)	4/11 (36%)
Total	6/12 (50%)	4/10 (40%)	10/22 (45%)

No effect of synchronization protocol (P = 0.64), semen extender (P = 0.33), or synchronization-by-extender interaction (P = 0.83).

3.4. Discussion

This study demonstrated the first evidence that pre-treating semen with CC in egg yolk-free Tris-glycerol extender provided adequate protection to beef and bison sperm against cryodamage that ultimately yielded fertility *in vivo*. We achieved pregnancy rates of up to 60% in multiparous beef cows and 36% in wood bison following fixed time artificial insemination with

CC-TG extender (egg yolk-free). Cryopreservation of bison bull semen using high concentration of CC (2 mg/mL semen) caused sperm plasma membrane changes leading to failure in fertility. When lower concentration of CC (1 mg/mL semen) was used, beef and bison sperm survived cryopreservation and yielded comparable fertility. This is the first report on the birth of live bison and beef calves following fixed-time artificial insemination using CC-TG extender. Cholesterol (delivered via cyclodextrins) may be used as a protein-free alternative to replace egg yolk in semen extenders for cryopreservation of beef and bison semen.

Cholesterol is known to stabilize plasma membrane and decrease sperm susceptibility to cold shock (Annabelle Darin-Bennett and White, 1977). The incorporation of cholesterol to the sperm membrane has been shown to increase linearly with CC concentration (Purdy and Graham, 2004b). Optimal CC concentration of 1.25 to 2.5 mg CC/100x10^6 sperm for beef sperm was previously suggested before dilution with egg yolk containing extender (Purdy and Graham, 2004a), as compared to 0.5 or 1 mg CC/100x10^6 sperm without egg yolk in the current study. This translates roughly to a 2.5-3 times increase in cholesterol concentration in the previous study as compared to an estimated 1.5-2 times in the current study. Increasing sperm membrane cholesterol concentration by 1.5-2 times raised cholesterol:phospholid ratio of bull sperm from 0.45 (Annabelle Darin-Bennett and White, 1977; Parks and Lynch, 1992) to 0.68-0.9 which is similar to that of rabbit (0.88) and human (0.99), species that are cold shock resistant (Annabelle Darin-Bennett and White, 1977). Therefore, bovine sperm incubated with exogenous cholesterol may be able to resist damage associated with cold shock.

Previous study conducted in our lab showed that although 2 mg CC-TG extended semen yielded post-thaw sperm motion characteristics comparable to TEYG extender, no pregnancy was achieved in bison females after fixed-time artificial insemination (Yang, Chapter 2). In the current study, poor *in vivo* fertility potential of 2 mg CC-TG extended semen was also observed in beef cows. Bull sperm viability began to decline with the addition of 3.75 mg CC/100x10^6 sperm while concentrations greater than 6.25 mg CC/100x10^6 sperm killed bull sperm before freezing (Purdy and Graham, 2004b). Although that 2 mg CC/100x10^6 is not sufficient to affect cell survival, we presume that sperm fertility was negatively impacted. Cholesterol depletion from plasma membrane initiates capacitation in mammalian sperm (Bailey, 2010; Visconti et al., 1999). Addition of exogenous cholesterol to sperm prevented capacitation and acrosome reaction

(Visconti et al. 1999; Zarintash and Cross, 1996). Therefore, we assumed that although treating sperm with 2 mg CC/mL did not affect its cryo-survival, issues associated with capacitation and acrosome reaction in the female reproductive tract may have resulted in the lack of pregnancy.

CC-TG extended semen appear to have suffered less membrane damage as compared to TEYG extender both at initial thawing and after incubation. However, contrary to fertility results, CC concentration did not have an effect on the plasma membrane and acrosome integrity. Successful fixed-time artificial insemination relies on proper timing between insemination and ovulation, with the sperm being readily available to fertilize the oocyte upon ovulation. Sperm treated with greater concentration of CC (2 mg CC/mL) may require longer time to undergo capacitation and acrosome reaction in the female tract. Inseminating earlier compared to conventional artificial insemination protocols improved *in vivo* fertility in pigs using cryopreserved CC treated semen (Cristina Tomás et al., 2013). If 2 mg CC-TG extender were to be used for bull semen cryopreservation, it may be beneficial to inseminate prior to GnRH treatment to allow more time for sperm to capacitate. On the contrary, VAP and VCL for semen in CC-TG extenders were consistently higher compared to TEYG extender. Greater VAP and VCL are indicative of sperm capacitation and subsequent hyperactivity (Verstegen et al., 2001). However, high velocities are likely due to reduced viscosity in CC-TG extenders, as compared with TEYG extender. After cryopreservation, a portion of sperm have been observed to be cryocapacitated at thawing (Cormier et al., 1997; Cormier and Bailey, 2003). However, in the current study, no difference in acrosome integrity was observed between sperm treated with or without LPC. It may be possible that cryocapacitation was minimized with our semen cryopreservation protocol.

Bull to bull variations were observed for CC-TG extenders in both sperm motion characteristics and field fertility. Although Bull C appears to have innate poor semen quality, the difference was more prominent with CC-TG extenders. Factors that constitute an animal as a good freezer are not well established but seminal plasma proteins have been implicated (Yeste, 2016). Egg yolk offer cryoprotective effects by replacing lost phospholipids (Quinn et al., 1980), preventing lipid efflux by binder of sperm proteins with low density lipoproteins (Bergeron et al., 2004), and providing anti-oxidants to reduce reactive oxygen species build up (Bergeron and Manjunath, 2006). Anti-oxidative effects of CC have been observed in cryopreservation of buffalo

semen (Lone et al., 2016). CC-TG extenders may lack the additional protective effects of egg yolk against binder of sperm proteins. More data, involving large number of bulls, are required to determine if CC concentration x bull interaction exists. It should be kept in mind that beef bulls in this study were procured from the field and they did not undergo intensive selection pressure, as commonly found in breeding bull studs in the industry. Bull to bull difference may be minimal for CC-TG extenders under a commercial semen production setting.

This study has demonstrated that CC alone can be successfully used to replace egg yolk from beef and bison semen cryopreservation procedures. Field fertility rates comparable to industry fixed time artificial insemination rates were achieved using CC-TG extenders. The CC concentration of 1 mg/mL semen should be optimal for beef and bison semen cryopreservation. Further studies will focus on the optimization of semen processing protocols using CC and conducting field fertility trials involving large number of bulls and cows with collaboration of industry partners.

3.5. Acknowledgements

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CHAPTER 4: GENERAL DISCUSSION AND RECOMMENDATIONS

The main goal of this thesis study was the development of an animal protein-free semen extender for cryopreservation of bison sperm by pre-treating semen with cholesterol-cyclodextrin (CC) complexes prior to dilution in Tris-citric acid glycerol (CC-TG) extender. In addition to similarities in reproductive anatomy and physiology, beef cattle were used as a model to investigate fertility-associated problems in bison using Frozen semen treated with CC. Specifically, sperm motion characteristics (total and progressive motilities, and average path, curvilinear and straight line velocities), and plasma and acrosome membrane integrities were examined. Most importantly, fertility potential, both *in vitro* and *in vivo*, of different semen extenders were determined.

Initial experiments were conducted in wood bison to reduce concerns over biosecurity and undefined composition by replacing egg yolk with CC from semen extenders. Post-thaw semen quality in CC-TG extender was as good as in TEYG extender (Table 2.1). VCL was reported to be greater in the egg yolk-free CC-TG treatment. This was likely due to low viscosity of CC-TG extender in the absence of egg yolk allowing sperm cells to travel faster. In a previous study conducted in our lab, pre-treatment of bison sperm with CC prior to dilution in TEYG extender improved their cryosurvival (Hussain et al., 2013). LDL fractions in egg yolk are attributed to the main protective component for sperm against cold shock by binding with Binder of SPerm (BSP) proteins to prevent premature efflux of cholesterol and phospholipids (Bailey, 2010; Pace and Graham, 1974). On the contrary, cholesterol plays an important role in stabilizing the plasma membrane during cooling to prevent phase transition changes (A Darin-Bennett and White, 1977). Species with low cholesterol to phospholipid ratio such as boar and stallion are high susceptible to damage associated with cold shock. Our results suggested that after treatment with CC, sperm membrane was sufficiently stabilized during cooling to prevent irreversible phase transition changes with the increased plasma membrane cholesterol concentration. To ensure that cold shock was prevented by cholesterol per se and not glycerol, an experiment was designed where addition of glycerolated portion of the extender was delayed until after cooling to 4 °C. In addition, glycerol can have cytotoxic effects on the sperm after prolonged exposure (Macías García et al., 2012; Pursel et al., 1978). However, prolonged incubation of bull semen in egg yolk extender with

glycerol have shown to improve post-thaw sperm quality (Anzar et al., 2011). Our results demonstrated that temperature of glycerol addition had no effect on sperm motion characteristics at 0 and 2 h post-thaw (Table 2.2). Due to procedural ease, we recommend that glycerol can be added at 22 °C, prior to cooling. The presence of BSP proteins have been implicated to play an important role in the cryosurvival of bison semen. Epididymal bison sperm froze significantly better compared to its electroejaculated counterpart in Andromed, a soybean lecithin based extender (Aurini et al., 2009; Lessard et al., 2009). Poor freezing capability of ejaculated bison semen in Andromed was likely due to the presence of BSP proteins. It appears that increased concentrations of cholesterol in the sperm membrane was able to prevent the damaging effects of BSP proteins and thus minimized the extent of cryocapacitation.

Follow up experiments to determine the fertility potential of semen in CC-TG extender were conducted. There were no differences in cleavage and blastocyst rates between extenders after heterologous *in vitro* fertilization (IVF) using abattoir derived cattle oocytes (Fig. 2.4). However, after fixed time artificial insemination (FTAI), 2 mg CC-TG extender yielded no conceptions compared to 43% pregnancy rate using TEYG extender. It was suspected that poor fertility *in vivo* was due to an issue with capacitation and/or spontaneous acrosome reaction. Therefore, an experiment was designed to investigate spontaneous acrosome reaction as a result of cryocapacitation. Removal of cholesterol from the plasma membrane is an initial step in the initiation of sperm capacitation (Bailey, 2010). Exogenous cholesterol have been reported to prevent acrosome reaction in human sperm (Renee J Zarintash and Cross, 1996). No differences in structural characteristics were observed between TEYG and 2 mg CC-TG extenders throughout the incubation period (Fig. 2.6). In addition, lack of pregnancies from using 2 mg CC-TG extender could be due to bison specific issue.

In the second series of experiments, bison species-specific effects and concentration of cholesterol in CC-TG extenders were examined. Due to similarities between cattle and bison in reproductive physiology and anatomy, the same technologies can be applied with minor to no modifications. An initial study was conducted in beef bulls to examine the cryosurvival of semen frozen with TEYG, 1 mg CC-TG or 2 mg CC-TG extender (Table 3.1). There were no differences in sperm motion characteristics among extender treatments between pre-freeze and post-thaw stages. Cholesterol concentration incorporated into the sperm membrane have shown to increase

linearly with CC concentration (Purdy and Graham, 2004b). It appears that lower concentration of cholesterol was sufficient to protect sperm from damage associated with cold shock. Flow cytometry experiments to determine plasma and acrosome integrity revealed that CC treated semen have reduced CPM/RACR population at both 4 and 6 h of incubation period (Fig 3.4). This confirmed that the addition of cholesterol reduced cryocapacitation. Lastly, FTAI was conducted in multiparous beef cows to determine the fertility potential between extenders. There were no difference in pregnancy rates between TEYG and 1 mg CC-TG extender whereas 2 mg CC-TG extender performed significantly worse (Table 3.2). It can be speculated that concentration of cholesterol was the key issue in the lack of pregnancy previously reported in bison. It has been suggested that higher concentrations of cholesterol might delay sperm capacitation process compared to conventional TEYG extenders leading to an mismatch with timing of insemination and ovulation (Salmon et al., 2017). When boar semen frozen in egg yolk extender with CC treatment was used in a FTAI procedure, fertility was improved by decreasing the time interval from 37 h to 30 h between hCG treatment and insemination in pigs, which allowed more time for sperm to undergo capacitation (Tomás et al., 2013).

A further reduction of CC concentration from 1 mg CC/mL semen to 0.5 mg CC/mL semen was tested in beef bulls. Previous study recommended that the optimal CC concentration to be added to egg yolk based extender was between 1.25 to 2.5 mg CC/mL (Purdy and Graham, 2004b) (Purdy and Graham, 2004b). We speculate that reduced concentrations of CC may be optimal to minimize sperm plasma membrane modification. Fresh egg yolk readily contains approximately 11.8 mg/g of cholesterol which translate to 2.45 mg/mL in conventional 20% egg yolk extenders used in the bull semen cryopreservation (Fenton and Sim, 1991; Pasin et al., 1998). It is likely that in the absence of cyclodextrins, cholesterol from egg yolk cannot be readily incorporated into the plasma membrane. In addition, cholesterol in egg yolk likely transferred to existing lipid droplets which prevents cholesterol incorporation into the sperm membrane. This phenomenon needs to be investigated. Therefore, high concentration of cholesterol present in egg yolk extender does little to protect the sperm. However, the exact loading and delivery mechanism for CC is not well understood. Comparable pregnancy rates between TEYG and 0.5 mg CC-TG extenders suggest that lower concentrations of CC is sufficient for providing adequate protection for beef semen. However, individual bull differences were found with CC-TG extender. There was a CC concentration effect where 0.5 mg CC-TG yielded poorer total motility in comparison to 1 mg CC-

TG in 2 out of 3 bulls. The existence of "good" and "poor" freezers in the beef livestock industry is well known and bull selection may be a main contributor. Low number bulls available did not allow a proper investigation into bull to bull differences with regards to CC concentration. In addition, low number of animals inseminated lack the statistical power to properly differentiate these differences. Large field studies will be required to further investigate into these issues.

Based on the CC concentration studies in beef cattle, 0.5 mg and 1 mg CC-TG extender was tested in bison. We, for the first time, achieved 4/11 (36%) pregnancy rate using 1 mg CC-TG extended frozen semen. Cryopreservation of bison semen in egg yolk-free semen extender was successful. Similar to beef cattle, semen frozen in 0.5 mg CC-TG extender resulted in poor total and progressive motilities compared to TEYG and 1 mg CC-TG extender (Table 3.6). It is likely that 0.5 mg CC-TG may be the minimal concentration required to protect sperm from cold shock while still retaining good fertilization potential. However, FTAI using 0.5 mg CC-TG extender in bison have not yet been conducted and requires further investigation. The potential for bison species specific effect still remains where bison membrane cholesterol:phospholipid ratio could be different to beef cattle and will respond differently to CC treatment.

The main goal of this research study was the development of an animal protein-free extender by using exogenous cholesterol as a replacement for use in bison. Based on the results of the study, 1) exogenous cholesterol (delivered via cyclodextrins) can be used to effectively replace egg yolk from semen extenders for cryopreservation of bison semen; 2) High concentration of cholesterol (2 mg CC/mL semen) have detrimental effects on sperm's fertilizing potential; 3) Pre-treatment of sperm with lower concentrations (0.5 and 1 mg CC/mL semen) of cholesterol in egg yolk-free extender are recommended to cryopreserve beef and bison semen, respectively.

Successful development of an animal protein-free semen extender can have huge implications on conservation efforts of wood bison population in Canada as well as for bovine AI industry. Tuberculosis and brucellosis plague Wood Buffalo National Park where much of the wood bison's genetic diversity resides. Harvesting gametes and propagating disease-free bison herds may be the most cost effective protocol to prevent the loss of genetic diversity. Washing procedures as recommended by the IETS can be effective in removing brucellosis from bison oocytes. Bison semen can also be washed to be free of bacteria through centrifugation through a density gradient. Development of a bio-secure semen extender will ensure that no diseases can be

transmitted through the usage of frozen semen. This can assist the effectiveness of other ARTs such as artificial insemination, *in vitro* fertilization and embryo transfer.

Perhaps the largest benefit of animal protein-free semen can be observed in improving production in the bison and cattle industry. International import and export of bovine semen have stringent requirements that emphasize on the importance of pathogen-free semen diluents (CFIA, 2002; OIE, 2016). Additionally, the cost of CC extender per semen dose is minimal. Since bison require longer period of time to reach maturity and slaughter conditions, there is a need to increase efficiency of production and herd genetics. The majority of bison farms currently utilizes herd males for natural breeding. Bison, unlike domestic cattle, take considerably longer period of time to reach sexual maturity i.e. around 2 years of age. However, due to herd dynamics, younger males may not have access to the females until they are much older. Application of FTAI in bison operations not only reduce the costs associated with keeping non-reproductive males but allow more efficient management of the herd. In addition, generation gap could be reduced to speed up genetic selection by utilizing genetics from younger males. With access to clean semen from diseased herds, bison producers can vastly improve production yields and economic gain. The majority of AI conducted in the cattle industry is in dairy cows whereas beef producers tend to lean towards natural breeding on pasture. However, we argue that the implementation of FTAI protocol can vastly increase both the efficiency of production and herd management in beef cattle. FTAI allows producers to select from the improved genetics each year without having to purchase a new bull, in addition to having a tighter calving season. The amount of animal handling in beef cattle can be limited to 3 days within an 8 day period which can be easily achieved. In addition, with the use of CC-TG extender, semen can be exported and imported worldwide with less concerns over biosecurity.

However, several factors should be taken into account with the application of CC-TG extended semen for FTAI in the future. Current CC-TG semen processing procedures consist of a step-wise dilution. Initially, diluted semen is treated with CC. After a brief incubation period, glycerolated portion of the extender is added and the extended semen is ready to be cooled and frozen. This can be a cumbersome process and is not as easy to conduct compared to the one step dilutions with most commercially available semen extenders. The step-wise procedure is normally required when CC is added before dilution in egg yolk extender. CC is required to co-incubate

with sperm in a lipid-free medium or lipids will interfere with incorporation of cholesterol into the sperm plasma membrane (Purdy and Graham, 2004b). However, addition of CC directly with glycerolated component may be feasible in the absence of egg yolk which would increase the ease of use. This could potentially mean that CC-TG extenders can be readily usable in a single step dilution once all components are combined together. Glycerol have shown to have an affinity to bind to the equatorial regions of cyclodextrins and could possibly interfere with the protective actions of CC (Bastos et al., 1997; Moreira and Bastos, 2000). However, the mechanisms and effects of glycerol to CC interactions are still unclear and will require further investigation. It is likely that the contradicting effects of glycerol are negligible.

Mechanism of CC delivering cholesterol to the plasma membrane still requires investigation. In previous studies, CC have been referred to as cholesterol-loaded cyclodextrin. However, loading mechanics for CC are not well understood. The protective properties of CC have mainly been contributed to its ability to maintain membrane stability. Other studies have demonstrated that CC may also have antioxidative effects (Lone et al., 2016; Naseer et al., 2015). Characterization and localization of cholesterol incorporated in to the membrane can provide the initial insight as on how cholesterol act to protect sperm from cold shock. Cholesterol is mainly concentrated at lipid rafts regions within the plasma membrane. It is yet unclear whether cholesterol from CC is also concentrated in lipid rafts or there is an equal distribution of cholesterol across the plasma membrane. In addition, it would be critical to determine the optimal concentration of CC for use for both cattle and bison. Individual species differences are expected due to varied membrane cholesterol to phospholipid ratio. Our experiments suggest that bison will require higher concentration as compared to cattle but lower than 2 mg CC concentration to retain good field fertility. Although not studied, bison likely have lower membrane cholesterol:phospholipid ratio in comparison to domestic cattle.

A major weakness of this study was the low number of animals utilized, particularly for bison. Large scale field fertility trials are necessary to fully examine the efficacy of CC-TG extenders. Bulls of varied genetic background should also be tested to determine if CC-TG extender can raise bull specific issues. CC-TG extender does not appear to be as effective as TEYG extender for certain animals and caution should be exercised with known poorer freezers. However, concentration of CC could be modified individually as an attempt to improve

freezability for poor freezing bulls. With regards to bison, it is critical to note that bison are wild animals and each handling involves large amounts of handling stress on them. Minimum handling during FTAI will be crucial for commercial acceptance and application. Additional barriers to widespread application of ARTs in bison are the requirements for specialized restraining systems including hydraulic squeeze chute and trained personnel. However, the economic and conservation benefits from application of ARTs in bison may outweigh the costs and potentially create new opportunities in the industry. Successful development of animal protein-free semen extender is critical to avoid the transmission of diseases and will provide new avenues for the migration of male genetics previously inaccessible. This new procedure will have important applications to the livestock industry and conservation efforts extending to plains bison and possibly other wild bovid.

In conclusion, this study demonstrated that exogenous cholesterol, delivered by cholesterol-cyclodextrin complexes, can successfully replace egg yolk in semen extenders for cattle and bison. CC concentrations of 0.5 and 1 mg CC/mL semen appear to be most beneficial to yield high percentage of motile sperm post-thaw and *in vivo* fertility following FTAI. Higher concentrations of CC (2 mg CC/mL semen) performed poorly in *in vivo* studies. Individual bull and species differences to CC-TG appear to exist and will require further investigation. However, in most cases, CC-TG extender can act as a biosecure and defined composition alternative to egg yolk extenders for cryopreservation of bison and beef semen.

CHAPTER 5: REFERENCES

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